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CONTENTS.

No. 1, July 1, 1916.

No. 2, August 1, 1916.

No. 3, September 1, 1916.

No. 4, October 1, 1916.

No. 5, NOVEMBER 1, 1916.

THE EFFECT OF ADULT CHICKEN ORGAN GRAFTS ON THE CHICK EMBRYO.

By JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 TO 5.

(Received for publication, May 1, 1916.)

In the course of experiments on the factors of resistance to heteroplastic tissue grafts in the chick embryo, observations were made on the effects of certain organ grafts on the embryo itself.[1] The working out of the finer histological details of this process has been taken up, at my suggestion, by Dr. Vera Danchakoff. This brief note on the original observations is published now for completeness and record.

Experiments.—On the 7th day of incubation openings were made in the shells of hens' eggs containing embryos, and small amounts of finely divided adult organs were deposited on the outer membrane (allantois and chorion). After this procedure the eggs were closed and sealed with paraffin and then returned to the incubator. On the 17th or 18th day of incubation the eggs were opened for examination. This constituted a control for the experiments on heteroplastic grafting, where, in the main experiment, grafts of heterologous tissues were introduced with an addition of grafts of homologous adult organs.[2] As there were a comparatively small number of eggs with the organ grafts alone in each experiment and as the technique throughout the series was the same, the results as a whole will be considered rather than the individual experiments. The adult chicken tissues used for the experiments were spleen, liver, kidney, bone marrow, pancreas, bone, and muscle. With the exception of pancreas, which caused wide-spread digestion of the embryonic tissues, all the tissues survived the grafting. As only spleen, liver,

[1] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

[2] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

kidney, and bone marrow caused other than local changes, the behavior of the other tissues will be disregarded in this communication.

Membranes.—On opening the eggs on the 18th day of incubation, 11 days after the introduction of a graft of one of the various organs, it was found that aside from the local changes about the graft in the outer membrane, there were scattered through the various membranes small, gray, semitranslucent nodules varying in size from a pin-point to a pin-head (Fig. 1). The majority of these contained in the center a small, firm, white dot. This change in the various membranes seemed to be no more frequent near than at a distance from the graft and occurred in other membranes of the embryo than that supporting the graft. There was considerable variation in the frequency with which this condition was observed. Its most common association was with spleen and bone marrow grafts, but it also occurred in typical form in association with the liver, and, still less frequently, with the kidney.

Histologically the nodules were made up of a collection of cells which showed every gradation from the mother cell of both the granular and non-granular white cells of the blood to the typical adult type. Every stage was found from a small clump (Fig. 2) of the former to a large dense mass with a necrotic center (Fig. 3). Immediately around the central dead area in these instances was a layer of multinuclear giant cells. Between this layer and the remaining part of the nodule was generally a zone of loose connective tissue. The outer portion was composed of a dense layer of cells of the character mentioned above.

Spleen.—Next to this change in the membranes, the most striking and constant change, after grafting with the adult organs, was observed in the embryo spleen. The spleen of the normal 18 day chick embryo is a small pinkish white organ, little larger than the head of a common pin. In embryos which bore adult organ grafts, the spleens were found to be greatly altered. This change varied considerably in extent. In some of the embryos the spleens were only slightly enlarged but showed marked congestion, and in others they were even hemorrhagic. In the extreme cases the spleen was greatly enlarged and many times the size of that seen in the normal animal (Fig. 4). The larger spleens showed numerous firm white

nodules which on the cut surface stood up above the spleen substance and presented a dry chalky appearance (Fig. 5).

The finer histology of the changes observed in the spleens, has been made the subject of a special study by Dr. Danchakoff. Hence it may suffice to say that while the spleen of a normal embryo of this age presents only a beginning differentiation of the cells, after grafting this process is well advanced and numerous cells of both the granular and non-granular type are found (Figs. 6 and 7). The firm white nodules are similar to those seen in the membranes. They consist of areas of necrosis surrounded by multinuclear giant cells with a layer of loose connective tissue separating them from the spleen substance.

Skin and Subcutaneous Tissue.—In a small proportion of embryos showing the above changes in the membranes and spleen, gross changes occurred also in the skin and subcutaneous tissues. The most marked example of this kind was from a liver graft in the embryo of which the same grayish semitranslucent nodules were scattered through the skin and subcutaneous tissues As in the other instances, the centers were frequently white and opaque (Fig. 8). Changes of this kind were perhaps more frequent than our material would suggest, but sections of the skin were taken only when macroscopic nodules were present. The second most striking instance of this kind resulted from an adult spleen graft. This embryo showed a nodule of considerable size in the skin of the neck, and underlying this several larger masses (Fig. 9). Microscopically the nodules resembled those seen in the membranes.

Other Organ Changes.—In the liver and kidneys of the embryos clumps of cells were observed of the same type as those described above. They occurred in masses around the vessels (Figs. 10 and 11), but as a rule were not so dense as those seen in the other tissues and were free from necrosis. These changes were found in the liver and kidneys, resulting from grafts of all the tissues mentioned above; namely, spleen, bone marrow, liver, and kidney.

DISCUSSION AND SUMMARY.

The explanation of the wide-spread changes in the embryo after grafting with adult organs offers a problem of considerable interest. Just what factors are responsible for them we are unprepared to say. They occur only after the grafting of certain adult homologous organs and are absent after grafting with such tissues as the chicken sarcoma, adult muscles, bone, and cartilage. Likewise no systemic changes have been observed after grafting of various tissues from foreign species, such as rat and mouse embryos, rat and mouse tumors of various sorts, or the normal organs of an adult animal of a foreign species. Whether or not this extensive proliferation of the white blood cell elements is to be looked on as a stimulation of a center with metastasis in the membranes and organs, or whether it is a general stimulation to the anlage of the various cells, is a point difficult to determine. Judging from the distribution and character of the proliferation, the latter seems the more plausible view. Moreover, the reaction may be of the same type as that observed by Da Fano[3] in mice after immunization against cancer by means of tissue injections. The changes he describes, however, were not so pronounced in character and were for the most part confined to the plasma cell in the connective tissues. The possibility of the action of infection must also be considered. Smears from the embryo spleen and the scattered nodules failed, however, to show microorganisms, and cultures taken on the ordinary media were negative for bacteria. If the reaction was a result of bacterial invasion, we should expect to find the changes occurring after the implantation of some of the transplantable rat and mouse tumors, for it is well known that they become highly contaminated. But such is not the case. A possible explanation of the necrosis is that the nutrition supply does not keep pace with the growth, so that a condition develops analogous to that of the central necrosis in rapidly growing tumors. We conclude, therefore, that grafts of adult spleen, bone marrow, liver, and kidney placed in the outer membrane of a chick embryo cause stimulation of the embryo spleen and lead to proliferation of certain leukocytic elements in the mesoderm, subcutaneous tissues, and around vessels in the liver and kidney.

[3] Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. Outer membrane of an 18 day old chick embryo inoculated with adult chicken liver on the 7th day. This shows the numerous white nodules scattered throughout the entire membrane.

Fig. 2. A small cell collection in the outer membrane of an 18 day old chick embryo after grafting with adult kidney on the 7th day of incubation. *a*, non-granular cells; *b*, granular cells.

Fig. 3. Section of a larger nodule in the embryonic membranes of a chick embryo which had received an adult spleen graft on the 7th day of incubation. *a*, dense zone of cells; *b*, loose fibrous tissue; *c*, layer of multinuclear giant cells; *d*, necrotic center.

Plate 2.

Fig. 4. Comparison of the spleens from normal 18 day old chick embryos (*a*) with the spleens from 18 day old embryos which had received adult organ grafts on the 7th day.

Fig. 5. A large nodular spleen (*Sp.*) in an 18 day old chick embryo which had received an adult liver graft in the outer membranes on the 7th day of incubation. (*St.*, stomach.)

Plate 3.

Fig. 6. Section of a normal spleen from an 18 day old chick embryo, showing only beginning differentiation of the cells.

Fig. 7. Spleen from an 18 day old chick embryo which had been inoculated in the outer membrane on the 7th day of incubation with adult liver. *a*, non-granular cells; *b*, granular cells.

Plate 4.

Fig. 8. Section from the toe of an 18 day old chick embryo showing cellular masses in the skin (*a*), and in the loose subcutaneous tissues (*b*). The latter shows the central area of necrosis. This embryo had had a graft of adult liver on the 7th day of incubation.

Fig. 9. Section of skin from an 18 day old chick embryo showing a nodule in the skin (*a*), and another in the subcutaneous tissues (*b*). This embryo received an adult spleen graft on the 7th day of incubation.

Plate 5.

Fig. 10. Kidney from an 18 day old chick embryo, showing cell collections about the vessels (*a*). A spleen graft had been introduced into the outer membrane on the 7th day of incubation.

Fig. 11. The liver from the same embryo described in Fig. 10, showing the same type of cell collections about the vessels (*a*).

IMMUNITY FACTORS IN PNEUMOCOCCUS INFECTION IN THE DOG.

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PLATE 6.

(Received for publication, April 24, 1916.)

The varied native resistance that different animal species, or individuals of the same species, offer to bacterial infection has been the object of much investigation and also of speculation. This has naturally arisen from the *a priori* fact that a thorough knowledge of nature's methods of combating infections is an indispensable prerequisite of a successful treatment of infectious diseases. In our opinion, a study of the problem can be successfully approached by determining the fate of bacteria when inoculated into animals possessing widely different degrees of resistance.

Dogs have a comparatively high resistance to pneumococci, while rabbits succumb to an exceedingly small quantity of a virulent culture. When pneumococci are introduced into the circulation of rabbits, they soon begin to multiply and a fatal septicemia rapidly follows. Just what takes place in dogs under similar conditions has not been definitely determined and the mechanism of their high resistance is unknown. Hence, it was believed that a detailed investigation of the phenomena following intravenous injections of pneumococci in dogs might yield a fuller knowledge of infection and resistance in general.

Previous Observations.

Fraenkel states that dogs are quite refractory to intravenous inoculations of pneumococci.[1] Gamaléia claims that subcutaneous inoculations cause only local reactions while intrathoracic inoculations give rise to a lobar pneumonia similar

[1] Fraenkel, A., *Z. klin. Med.*, 1886, x, 401.

to that seen in man.[2] Monti showed that subdural injections of pneumococci produced typical meningitis in dogs.[3] It is claimed by Kruse and Pansini that subcutaneous inoculations of from 2 to 3 cc. of culture killed dogs within 2 to 5 days, while intravenous and intraperitoneal inoculations were without effect.[4] In a study of intrabronchial insufflations of pneumococci in dogs Lamar and Meltzer[5] observed that dogs receiving large quantities of culture developed persistent fever, did not eat, were prostrated, and died 2 to 4 days after the inoculation. These dogs gave positive blood cultures at autopsy but no detailed observations were made on the course of the septicemia. Wollstein and Meltzer[6] noted, moreover, that intrabronchial insufflations of virulent pneumococci gave rise to a bacteremia, while non-virulent cultures did not. Here, also, no quantitative observations were made.

From the literature it is readily seen that there is little definite knowledge concerning the fate of pneumococci injected into the circulation of dogs, and also that no systematic study has been made of the phenomena that the injections may incite.

The object of the present study was (1) to determine more definitely the relative resistance of dogs to pneumococcic infection, (2) to follow quantitatively the fate of the injected bacteria from the beginning to the end of the infection, and (3) to investigate the defensive mechanism and immunity responses on the part of the infected animals.

Technique.

Dogs were given intravenous inoculations of from 1 to 4 cc. per kilo of a bouillon culture of virulent[7] pneumococci. Cultures were made from the heart's blood as early as 1 minute after the injection and at short intervals for the first 6 hours and then at 24 hour periods. The blood was obtained from the heart and the cultures were made according to the method described in connection with making blood cultures from rabbits infected with pneumococci.[8] After the course

[2] Gamaléia, N., *Ann. Inst. Pasteur,* 1888, ii, 440.

[3] Monti, A., *Riforma med.,* 1889, v, 344, 350.

[4] Kruse, W., and Pansini, S., *Z. Hyg.,* 1892, xi, 279.

[5] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.,* 1912, xv, 133.

[6] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.,* 1913, xvii, 353.

[7] The pneumococci were so virulent for rabbits that 0.00005 cc. killed within 24 hours. The virulence was maintained at this level by frequent passage in these animals.

[8] Bull, C. G., *J. Exp. Med.,* 1915, xxii, 457.

of the infections had been established in a general way, it was found that countable plates could be made with undiluted blood after the first hour, hence measured quantities of blood were put directly into the plates and melted agar was poured in before clotting occurred. At least three plates were made from each specimen of blood and the average figures recorded in terms of the number of colonies developing from 1 cc. of blood.

Course of the Septicemia.

Through the above procedure it was discovered that the curve of the blood cultures following intravenous inoculations of pneumococci in dogs is characteristic and presents several points of interest. The injected cocci leave the circulating blood rapidly. The mass of the bacteria disappears within the first 10 minutes, the cultures dropping from about 5,000,000 colonies per cc. 1 minute after the inoculation to 175,000 at 10 minutes after. From 3 to 6 hours after the inoculations the cultures show from 50 to 200 colonies per cc. of blood. At the end of 24 hours there is, as a rule, a slight increase over the 6 hour culture. At this point the septicemia may take one of two courses. The number of organisms may gradually decrease and the blood become sterile after 2 or 3 days, a true infection not arising. On the other hand, the septicemia may gradually increase for the next 24 or 48 hours and then rapidly ascend to a climax which is attained between the 4th and 5th days. At this point a downward direction is taken and the blood becomes sterile within from 1 to 4 days. This would probably be the end of the infection if it were not for the fact that a meningitis has invariably developed in the meantime. In a number of instances, however, the blood remained sterile in the presence of a severe, and, as a rule, fatal meningitis. Every fatal case had a severe pneumococcic meningitis. The following protocols illustrate the points mentioned.

Dog 1.—Weight 6 kilos. 6 cc. of a bouillon culture of pneumococci were injected into the ear vein and observations were made as given in Table I.

Autopsy.—The lungs are normal; the heart is not injured by the punctures; no hemorrhage into the pericardium. The spleen, liver, and kidneys are congested. There are abscesses in the medullary portion of both kidneys, and films show

TABLE I.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	4,000,000	
9 "	100,000	
2 hrs.	100	Vomiting and diarrhea.
6 "	60	Drowsy.
25 "	200	Sluggish; has not eaten.
49 "	4,000	Lethargic; does not eat.
72 "	5,000	" hyperesthetic.
96 "	22,000	" "
120 "	3,000	Lying in cage; "
144 "	700	Hyperesthetic.
168 "	5	Comatose.
180 "	0	Found dead.

many diplococci and polymorphonuclear leukocytes. The subdural spaces of the brain and cord contain pus, and films show numerous pneumococci and polymorphonuclear leukocytes. The pneumococci are evenly distributed and are not being phagocyted.

In this experiment (Dog 1) we have a typical representative of a large percentage of the successfully infected dogs. The culture made 1 minute after the inoculation gave 4,000,000 colonies per cc. of blood. The pneumococci left the circulation rapidly and at the 6th hour 1 cc. of blood gave only 60 colonies. The blood cultures take a similar course in every instance and for convenience this phase of the infection will be designated as the initial drop. The initial drop was followed by a gradual increase in the number of bacteria in the blood and at the 96th hour 1 cc. gave 22,000 colonies. This proved to be the height of the blood infection and was followed by a second abrupt drop, the blood being sterile at the 180th hour. The secondary drop in this instance reminds one of the termination of pneumonia in man by lysis. The septicemia was evidently not the cause of death in this case, since at the time of death the heart's blood was sterile. On the 3rd day the dog became hyperesthetic. The hyperesthesia gradually gave way to paralysis of the extremities and this was followed by coma. Opisthotonos was also present. The autopsy revealed a severe purulent meningitis.

Dog 2.—Weight 6.5 kilos. 13 cc. of a bouillon culture of pneumococci were given intravenously. The record is given in Table II.

TABLE II.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	7,250,000	
10 "	190,000	
7 hrs.	200	2 hrs. vomiting and diarrhea.
24 "	430	Sluggish; does not eat.
48 "	1,000	" " " "
72 "	6,000	Hypersensitive.
96 "	32,000	Definite symptoms of meningitis.
120 "	0	Lying in cage; cannot stand.
144 "	0	" " " " "
168 "	0	" " " " "
192 "	0	" " " " "
216 "	10	" " " " "
240 "	0	Eats; cannot stand. Gradually recovered.

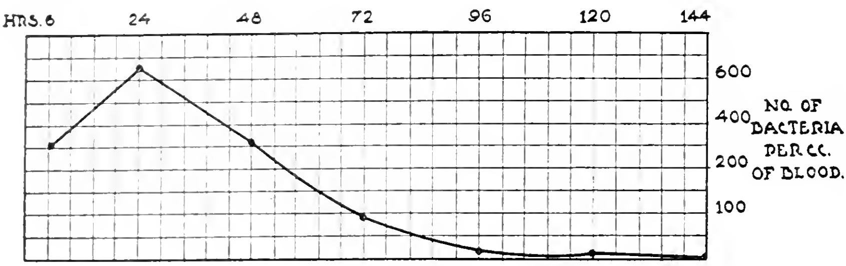
This dog differs from the first (No. 1) in that the secondary drop in the septicemia was of the nature of a crisis, going from 32,000 colonies per cc. of blood to sterility within 24 hours, with recovery. No relapse occurred in the septicemia, although a severe meningitis existed for several days. The pneumococci isolated on the 9th day grew in chains and were non-virulent (Fig. 1).

Dog 3.—Weight 7 kilos. 7 cc. of a bouillon culture of pneumococci were given intravenously. The record appears in Table III.

TABLE III.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	3,800,000	
10 "	80,000	
6 hrs.	75	2 hrs. vomiting and diarrhea.
24 "	140	Does not eat.
48 "	100	Eats.
72 "	75	Lively.
96 "	0	"
120 "	0	No further symptoms.

In Table III (Text-fig. 1) is given the course of the bacteremia when no infection develops. The initial drop occurs as in other cases. There is a slight return from the 24th to the 48th hour, but, as a rule, the blood becomes sterile before the 5th day and no further symptoms develop.



TEXT-FIG. 1. Composite curve of ten dogs that did not become infected. They were given intravenously from 1 to 3 cc. of a bouillon culture of virulent pneumococci per kilo of body weight. The abscissæ represent in hours the time after the inoculation; the ordinates, the number of pneumococci per cc. of heart's blood.

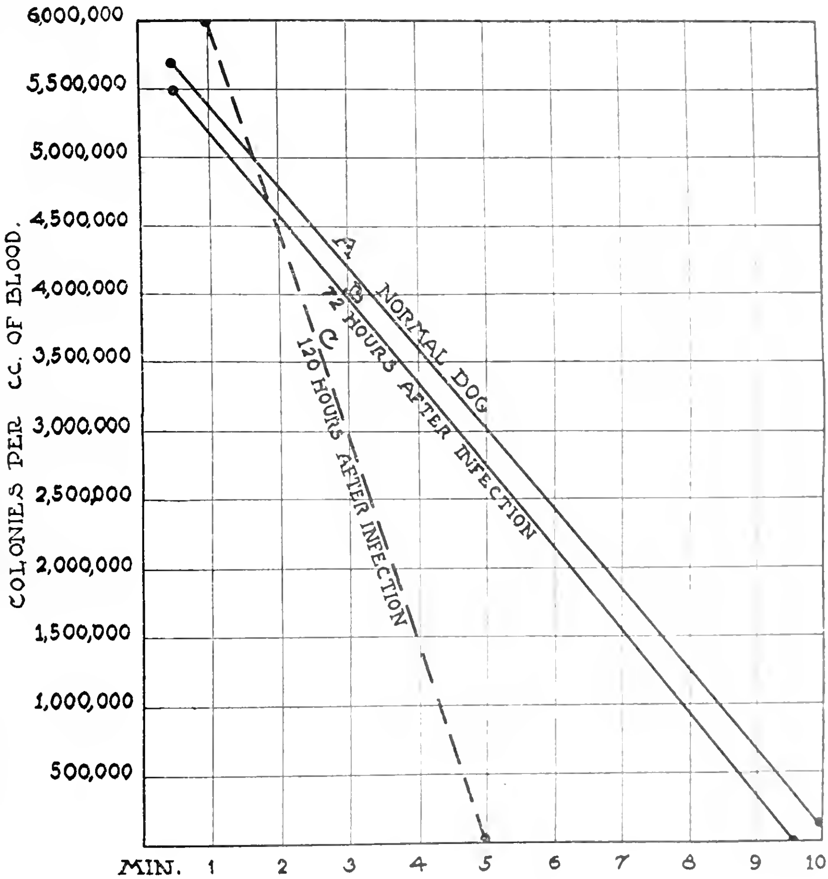
Dog 4.—Weight 6 kilos. 12 cc. of a bouillon culture of pneumococci were injected into the ear vein (Table IV).

TABLE IV.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	6,500,000	
6 hrs.	300	Drowsy.
24 "	1,500	Sluggish.
48 "	7,000	"
72 "	10,000	"
96 "	20,000	Meningeal symptoms.
120 "	4,000	Very sick.
144 "	1,300	Cannot stand.
168 "	5,000	Legs paralyzed.
192 "	35,000	Comatose.
216 "	50,000	"
240 "	110,000	"
255 "	25,000	Found dead.

Autopsy.—A severe purulent meningitis.

This protocol (Dog 4) is given because of the relapse of the septicemia. It is possible that this was due to an invasion of the blood from the meningeal infection.



TEXT-FIG. 2. Curves A, B, and C represent the rate at which the pneumococci left the circulation of dogs at the time of infection and at 72 and 120 hours after the infection, respectively.

The curves (Text-figs. 1, 2, and 3) of the blood infections as presented in the protocols have three points of especial interest: the initial drop, the ascension of the septicemia, and the secondary drop. Several questions arise concerning the nature of the forces operating

during these three phases. First, why do the blood cultures fall so rapidly immediately after the injections are made? Why does the septicemia begin first after 24 or 48 hours instead of directly after the inoculations? Are natural antibodies present and do they destroy the mass of the pneumococci, then become bound or exhausted and permit the few bacteria that escaped destruction to multiply and produce the septicemia? Or, on the other hand, may the natural antibodies not be exhausted, the fittest organisms survive and later become immune to the forces that destroyed the mass? As to the secondary drop it may be assumed as a working hypothesis that the blood suddenly acquires new forces that destroy the pneumococci or remove them from the circulation. These points are considered individually in the following pages.

Initial Drop.

Observations made in following the course of typhoid bacilli when intravenously injected into normal rabbits[9] and the effect of immune serum on pneumococcic septicemia in rabbits[8] led us to suspect that the pneumococci are agglutinated in normal dogs immediately after reaching the circulation. Indeed, experiments proved this to be the case. Pneumococci from 100 to 150 cc. of bouillon culture were injected into the ear vein and specimens of blood were taken from the heart at short intervals from the 1st to the 10th minute and examined for clumps. The observations are shown in Table V and Figs. 2 and 3.

TABLE V.

Time of taking specimen of blood.	No. of free bacteria.	No. of clumps.
min.		
1	Many.	No clumps.
2	Fewer.	Several.
3	Still fewer.	Many.
4	" "	Fewer.
5	" "	Still fewer.
6	" "	" "
7	None.	" "
8	"	" "
9	"	None found.

[9] Bull, *J. Exp. Med.*, 1915, xxii, 475.

The dog died on the 4th day.

Autopsy.—Lungs hemorrhagic; pleurisy with effusion; many pneumococci in the fluid; kidneys hemorrhagic; abscesses in the medullæ; severe meningitis.

The character of the clumps here observed differs from those formed in rabbits treated with immune serum in that they are much less compact. The agglutination proceeds more slowly also. The degree of agglutination is, however, directly proportional to the rapidity with which the blood cultures decrease. By the cultural method it was demonstrated merely that the number of viable bacteria speedily decreases immediately after the inoculations. The microscopic observations demonstrated, moreover, that the reduction in the cultures is due to the absence of the bacteria and that it is not a question of viability. An examination of the organs gave further proof of this since clumps of the bacteria were readily found.

The initial drop, then, is due to the agglutination of the pneumococci within the circulation and to the accumulation of the clumps in the lungs, liver, spleen, and other organs.

Secondary Drop.

Since the initial drop is the result of an agglutination of the pneumococci, it was logical to suppose that the secondary drop was due to a similar process. Yet, in order to explain the removal of the bacteria which had invaded the blood despite the presence of the natural agglutinins, or possibly after they had been exhausted, it was necessary to assume either that newly acquired agglutinins appear or that the pneumococci suddenly became more susceptible to the previously existing agglutinins. Hence experiments were devised to test these points.

Specimens of blood were taken from a series of dogs and the dogs were then inoculated with pneumococci in the usual way. The sera obtained before the inoculation and specimens taken each succeeding day were tested *in vitro* for agglutinins and opsonins. Neither agglutinins nor opsonins could be thus demonstrated before the 6th or 7th day. This was 48 hours after the break in the septicemia, and hence the assumption that these antibodies were operative in the animals at the time of the crisis in the septicemia seemed not to be

justified. It was thought, however, that the test could be made successfully in the animal body by determining the extent of agglutination and rate of disappearance of pneumococci injected at the time of the crisis. Thus, it was found that agglutination is more pronounced and that the bacteria leave the circulation much more rapidly at this time than even in normal dogs (Text-fig. 2). Hence a wide disparity exists between the demonstration of certain antibodies *in vitro* and *in vivo*. Representative experiments of this type are given in the protocols of Dogs 5 and 6.

Dog 5.—Weight 4.75 kilos. 14 cc. of a bouillon culture of pneumococci were given intravenously. On the 5th day after the inoculation the pneumococci from 150 cc. of a bouillon culture were injected into the ear vein, and cultures and smears were made from the heart's blood at short intervals, from the 1st to the 10th minute after the inoculation. Cultures: 1 minute, 210,000,000 per cc. of blood; 9 minutes, 2,000,000 per cc. Films: 1 minute, many free diplococci and some clumps; 3 minutes, many clumps and few free bacteria.

Dog 6.—Weight 7.75 kilos. 18 cc. of a bouillon culture of pneumococci were given intravenously. On the 5th day 10 cc. of culture were injected into the ear vein and cultures were made from the heart's blood at 1 and 8 minutes respectively. Cultures: 1 minute, 7,840,000 colonies per cc. of blood; 8 minutes, 12,500.

By comparing the rapidity with which the bacteria leave the circulation of normal dogs with that of Dogs 5 and 6, it is readily seen that the disappearance is more abrupt and complete in the latter. In normal dogs about one-twentieth of the cocci injected were still present at the expiration of 10 minutes, while in the dogs injected on the 5th day of the infection only from $\frac{1}{200}$ to $\frac{1}{600}$ of the injected bacteria were present at the expiration of 8 minutes. Since the rate at which bacteria leave the circulation of an animal is directly proportional to the extent of agglutination, we have here corroborative evidence of the microscopic finding that pneumococci are much more actively agglutinated in infected dogs at the time of the crisis in the septicemia than in normal dogs.

The results of these experiments have two points of unusual interest. First, it is shown that bacterial antibodies are effective in the animal body before they can be demonstrated *in vitro*. Second, it is demonstrated that acquired antibodies are active at the time of a crisis in pneumococcic septicemia in dogs. These facts are doubly

interesting because they seem to shed light on the cause of the crisis in pneumonia in man. Acquired antibodies do not become demonstrable *in vitro* in the serum of pneumonia patients, as a rule, until some time after the crisis,[10] but in the light of the above findings one is justified in concluding that they are operative in the patients at the time of the crisis.

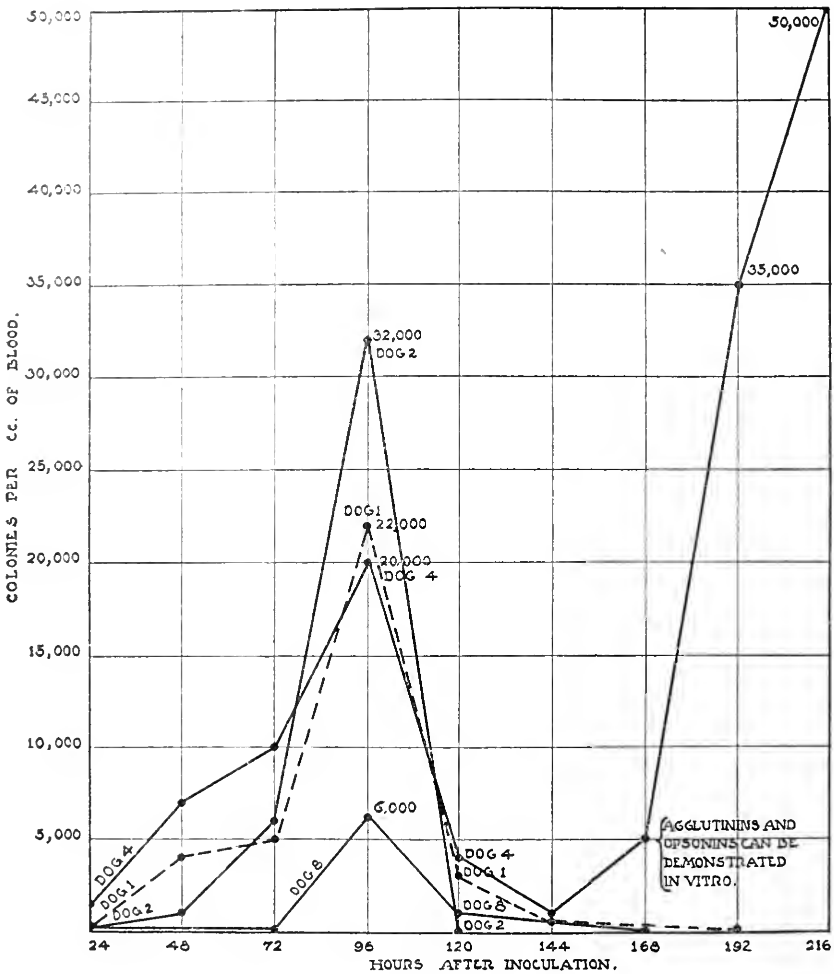
Ascension of the Septicemia.

After the initial drop the septicemia gradually increases for 48 hours and then rapidly rises to its apex (Text-fig. 3). Why a blood invasion is possible at this time, when the bacteria could not remain in the circulation at the time of the injection, is not immediately evident. Have the natural antibodies been exhausted or bound by the injected bacteria, or have the bacteria that escaped destruction become immune to the antibodies? It is possible that the septicemia is a resultant of two factors; a partial depression of the antibody activities and a lessened susceptibility on the part of the bacteria. The following experiments were made to study these points.

Bacteria.—Pneumococci were isolated from the heart's blood at each phase of the septicemia and kept on blood agar plates in the ice box until the infection had run its course. On the 6th day bouillon cultures were made from the different plates and the strains were tested for agglutination and opsonization with sera obtained from dogs on the 6th and 7th days of the infection. Thus it was found that the strains isolated as the septicemia was ascending were resistant to the action of the sera, while those isolated as the septicemia was declining were more readily opsonized and agglutinated than the original strain (Figs. 4 and 5). The resistant strains were both opsonized and agglutinated by a highly active immune horse serum, and the increased resistance disappeared after six transfers in bouillon.

Antibodies.—Dogs exhibiting a progressing septicemia were reinjected and the rate of disappearance of the newly injected pneumococci was determined.

[10] Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.



TEXT-FIG. 3. Crisis in septicemia.

Dog 7.—Weight 8.5 kilos. 24 cc. of a bouillon culture of pneumococci were given intravenously. For blood cultures and reinjection see Table VI.

TABLE VI.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	8,500,000	
6 hrs.	350	
24 "	600	
48 "	5,000	
		After the culture was made 8 cc. of culture were injected into the ear vein.
1 min. after reinjection.	3,500,000	
3 hrs. " "	3,000	
72 hrs.	14,500	
96 "	24,000	
120 "	Dog was found dead.	Meningitis.

The figures in Table VI show that even in the presence of a progressing septicemia freshly injected pneumococci rapidly leave the circulation (Text-fig. 2). Indeed, there were fewer bacteria in the heart's blood 3 hours after the second inoculation than immediately before. It was found that the number of pneumococci in the circulation 3 hours after the second injection was always in direct relation to the number present at the time the inoculation was made. As a rule, the cultures made at the expiration of 3 hours gave from 50 to 75 per cent as many colonies as those preceding the reinoculation. This held good although the dogs had infections ranging from 250 to 150,000 colonies per cc. of blood at the time they were reinoculated.

A question as to the identity of the residual pneumococci naturally arose. Are they the organisms of the septicemia, or a portion of the freshly injected culture? As already stated, cultures isolated during the ascension of the septicemia have been found to be resistant to the opsonins and agglutinins of weakly immune sera. Hence, if the residual pneumococci are the septicemic organisms, they should manifest this same resistance. This proved to be the case. Bouillon cultures were made with blood from the heart immediately before and 3 hours after the second inoculation and were tested on the following day for agglutination and opsonization with

serum taken from a dog on the 6th day after inoculation with pneumococci. The culture with which the dog was reinoculated was both opsonized and agglutinated, while the cultures obtained from the heart's blood immediately before and 3 hours after the inoculation were neither opsonized nor agglutinated.

An interpretation of the different phases of the blood infection is now possible. The initial drop is the result of the agglutination of the pneumococci in the circulation of the dogs and the accumulation of the clumps in the organs. After an incubation period of from 24 to 48 hours the pneumococci become resistant to the action of the agglutinins and possibly other antibodies and reinvade the blood stream. But before the septicemia can become fatal it is halted by the appearance of newly formed antibodies.

Severity of the Meningitis and Phagocytosis.—As previously stated, every fatal infection was accompanied by meningeal symptoms and a purulent pneumococcic meningitis was found at autopsy. A few dogs recovered after a prolonged attack of meningitis. Others, having lighter attacks and showing signs of improvement, were killed in order to study the phenomena that accompany improvement or recovery.

Films and sections from the brain and cord of the fatal cases showed large numbers of pneumococci and polymorphonuclear leukocytes. The pneumococci were evenly distributed and little or no phagocytosis had taken place. Similar preparations from dogs killed while in a state of improvement gave a different picture. The pneumococci were more or less collected into clumps and many leukocytes contained large numbers of bacteria (Figs. 6 and 7).

DISCUSSION.

The facts established in the above experiments are interesting when considered in connection with many obscure points in the epidemiology and course of infectious diseases. First, it has been shown that bacteria isolated at different stages of an infection vary in their susceptibility to specific antibodies and in their infecting power. This recalls the fact that many infectious diseases are more readily transmitted from person to person during the active phase of the dis-

ease than in early convalescence. Epidemics arise, probably, because the infectious agent is passed at short intervals from individual to individual during the early stage of the disease and, therefore, before the agent has been weakened by immunity responses on the part of the individual. As soon as early and frequent contacts are avoided by isolation, the agent becomes less infectious and the epidemic subsides.

An incubation period of about 48 hours always preceded the pneumococcic septicemia in the dogs, although millions of the bacteria were injected directly into the circulation. The experiments devised to explain this delay have shown clearly that this time is necessary for the pneumococci to adapt themselves to their new and adverse environment. When they had once become immune to the injurious antibodies that existed, a rapid multiplication occurred and symptoms of disease became manifest. And if these facts can be used to interpret the incubation period of infectious diseases in man, the logical conclusion is that it has a similar meaning there, for if bacteria find ideal conditions for multiplication on entering a new host, only a few hours will elapse between the time of infection and the appearance of symptoms of disease.

It may at first seem paradoxical that the pneumococci became more resistant to the action of antibodies in the early part of the infection and then later more susceptible. This is probably due to the nature and strength of the antibodies operative at the different phases of the infection. In the incubation period, only the natural antibodies are present, while in the decline of the septicemia acquired antibodies have appeared. Corroborative observations have been made *in vitro*. Feiler found that typhoid bacilli grown in fresh normal rabbit serum become more resistant to the bactericidal action of the serum,[11] while Stryker found that growth in highly immune horse serum renders pneumococci more agglutinable and less virulent.[12] Whether bacteria become weaker or stronger in the presence of antibodies depends, no doubt, upon the relation between the plasticity and adaptability of the bacteria and the strength of the antibodies.

[11] Feiler, M., *Z. Immunitätsforsch., Orig.*, 1916, xxiv, 411.

[12] Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

The demonstration of the activity of newly acquired antibodies at the time of the crisis in the septicemia throws light upon the natural recovery from infections diseases in man. If the host can offer sufficient resistance to the invading bacteria to forestall a profound depression of the physiologic functions, or a fatal issue, until specific antibodies can be formed and mobilized, recovery will result. On the other hand, if the host offers little or no resistance to the bacteria at the time of infection and the incubation period is eliminated, the battle is lost before defensive antibodies can be formed. The latter condition is found in the case of pneumoccocic septicemia in rabbits and the former in pneumococcic septicemia in dogs.

The cases of meningitis offered an excellent opportunity to study the manner of the destruction of the bacteria in pneumococcic infections. Despite the fact that it has been demonstrated that agglutination, opsonization, and phagocytosis bring about the destruction of myriads of pneumococci in infected rabbits treated with immune serum,[13] it is still held that this has little or nothing to do with the destruction of the bacteria in cases of pneumonia in man.[14] One argument against the phagocytic theory is that phagocytosis is never found in the lungs of patients dying of pneumonia. What takes place in the cases that recover is, of course, undeterminable. The observations made on the acutely fatal and convalescent cases of pneumococcic meningitis in dogs indicate that the degree of phagocytosis is inversely proportional to the acuteness and severity of the infection. In the fatal cases there was very little phagocytosis, while in the convalescent cases the pneumococci were being actively phagocyted. The same condition may hold in pneumonia in man.

SUMMARY.

Intravenous inoculations of from 1 to 3 cc. per kilo of body weight of a bouillon culture of virulent pneumococci produce septicemia and meningitis in dogs.

The injected pneumococci leave the circulation rapidly, but begin to reinvade the blood from 24 to 48 hours later. The septicemia reaches its climax between the 4th and 5th days and then abruptly

[13] Bull, *J. Exp. Med.*, 1915, xxii, 466.

[14] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

declines, the blood becoming sterile within from 1 to 3 days after the height of the septicemia is reached.

The initial disappearance of the pneumococci from the circulation has been found to be due to agglutination of the diplococci in the blood stream and accumulation of the clumps in the lungs, liver, spleen, etc.

If the dogs are reinoculated during the ascension of the septicemia, the injected diplococci leave the circulation as rapidly as in normal dogs. Cultures isolated in this stage of the infection, both before and from 3 to 4 hours after the reinoculation, are resistant to the agglutinins and opsonins of immune sera that agglutinate and opsonize the cultures with which the dogs were originally infected. Thus it follows that the pneumococci are able to reinvade the circulation because they have acquired a fastness to the existing antibodies and not because the antibodies have been bound or exhausted.

By reinoculating dogs at the time of the crisis in the septicemia it has been shown that the agglutination of the pneumococci is more rapid and complete and that the diplococci leave the circulation much more rapidly than in normal dogs. Hence acquired antibodies are operative within the animals at this time although they cannot be demonstrated *in vitro* until from 24 to 48 hours later.

Pneumococci isolated as the infection is subsiding are more susceptible to the action of immune sera than the original cultures injected.

It is probable that all the dogs would have survived the infection if a meningitis had not developed.

In the acutely fatal cases of meningitis few pneumococci are phagocyted, while in the milder and convalescent cases much phagocytosis occurs.

It is suggested that the incubation period of infectious diseases is due to the fact that the infecting agents must become adapted to the adverse conditions encountered in the newly infected host before they can multiply sufficiently to produce the symptoms of disease. It is further suggested that epidemics may arise because the infectious agent is passed from person to person in the ascending stage of the disease and thus enters new hosts in a state of maximum resistance to the natural antibodies of such individuals. When early contacts

are avoided, epidemics tend to subside because the infectious agent is weakened by the action of acquired antibodies during the period of convalescence.

EXPLANATION OF PLATE 6.

FIG. 1. Pneumococci isolated from Dog 2 on the 9th day of infection. The film was made from a bouillon culture.

FIG. 2. A film from the heart's blood of a dog 30 seconds after a suspension of pneumococci had been injected.

FIG. 3. A clump of pneumococci from the heart's blood of the same dog 2 minutes after the bacteria were injected.

FIG. 4. Pneumococci, immune dog serum, and guinea pig leukocytes. The bacteria were isolated from a dog 72 hours after it had been infected with pneumococci. The serum was obtained from a dog on the 7th day of a pneumococcic infection.

FIG. 5. The same as Fig. 4 except that the pneumococci were from the strain with which the above dog was infected.

FIG. 6. A film from the brain surface of a dog dying of an acute pneumococcic meningitis.

FIG. 7. A film from the brain surface of a dog in the convalescent stage of a pneumococcic meningitis.

FURTHER OBSERVATIONS ON THE AGGLUTINATION OF BACTERIA IN VIVO.

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PLATE 7.

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In a previous paper[1] I reported the occurrence and the apparent significance of the agglutination of bacteria within the circulation of infected animals. The points brought out are briefly as follows: Virulent pneumococci, dysentery bacilli of the Shiga type, and virulent influenza bacilli do not agglutinate when injected into the circulation of normal rabbits; while typhoid bacilli, dysentery bacilli of the Flexner group, and non-virulent influenza bacilli are agglutinated immediately after entering the circulation of these animals. On the other hand, an intravenous injection of a small quantity of specific immune sera causes an instantaneous agglutination of pneumococci and Shiga dysentery bacilli in the circulation of infected rabbits. The bacterial clumps accumulate in the organs where they are phagocyted. It was suggested at that time that the power of the blood to cause agglutination determines, apparently, in large measure, whether the bacteria are to be promptly removed from the circulation and septicemia avoided or whether they are to remain there and produce a blood infection. Further observations have been made in this connection and are collected in this paper.

EXPERIMENTAL.

Active Immunity and Agglutination in Vivo.—As stated above, virulent pneumococci and Shiga dysentery bacilli do not agglutinate in the circulation of normal rabbits. It has been more recently observed that a strain of *Bacillus mucosus capsulatus*[2] recently isolated

[1] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 484.

[2] The author is indebted to Dr. Warren R. Sisson, of Baltimore, for this strain.

from a case of pneumonia did not agglutinate in normal rabbits. It has also been shown that pneumococci and Shiga dysentery bacilli were made readily agglutinable in normal rabbits by intravenous injections of specific immune horse serum. It was not certain, however, that these organisms would be agglutinated in actively immunized animals. Immune animals are highly resistant to infection with these bacteria and if agglutination *in vivo* plays an important part in the protection of animals against infection, it should occur in this instance. Hence rabbits were immunized with these organisms and *in vivo* agglutination tests were made. It was thus found that pneumococci, Shiga dysentery bacilli, and Friedländer bacilli are agglutinated immediately upon entering the blood stream of actively immunized animals. Indeed, the agglutination takes place so quickly and the clumps leave the circulation with such rapidity that no time can be lost in taking the blood from the heart if clumps are to be found. Only a few bacteria are left in the circulation at the end of the 1st minute.

That *Bacillus mucosus capsulatus* is agglutinated so readily in actively immune animals is especially interesting since this organism is agglutinated with difficulty *in vitro*. The sera of the rabbits used for the *in vivo* agglutination tests would not agglutinate the bacilli in dilutions above 1: 5 *in vitro*.

Agglutination in Normal Rabbits.—A number of bacteria have been tested for agglutination in normal rabbits. The bacterial suspensions used in the tests were obtained either by centrifuging large quantities of bouillon cultures or by washing the bacteria from solid media with salt solution. In order to avoid false results an effort was made to free the suspensions of clumps before they were injected. As a rule, however, one specimen of blood could be obtained after the bacteria were injected before agglutination occurred, and this eliminated the possibility of the clumps that were found in the later specimens having been injected. Bacteria that agglutinate spontaneously in fluid media or in salt solution suspensions do not give satisfactory results. Sufficient quantities of the cultures were injected to give a number of organisms in each microscopic field of a film from the heart's blood.

Non-Virulent Pneumococci.—A strain of pneumococci of low virulence was obtained from The Rockefeller Institute Hospital. It had been cultivated in 10 per cent immune horse serum for seventy

generations. The immune serum was removed by ten transfers in plain bouillon. Each transfer was made by inoculating 100 cc. of bouillon with 0.5 cc. of the previous culture. This procedure diluted the immune serum so highly that it could not have influenced any of the subsequent results. Even at this time 15 cc. of a 20 hour bouillon culture did not kill a 1,500 gm. rabbit.

With strains of the same organism differing so widely in virulence, it was interesting to compare the results of intravenous inoculations with the two strains. Virulent pneumococci, it will be remembered, remain in the circulation of normal rabbits, soon begin to multiply, and a fatal septicemia develops from an exceedingly small number of organisms. The virulent organisms are neither opsonized nor agglutinated by normal rabbit serum *in vitro* or *in vivo*. The fate of the non-virulent organisms is found to be as follows: Agglutination occurs immediately after the cocci reach the circulation, the clumps leave the blood stream with great rapidity and accumulate in the organs where they are taken up by phagocytic cells. In this case no infection arises and the rabbits remain in good condition. It is also interesting to point out that the two strains do not differ in toxicity; *i.e.*, when dissolved in bile salts, one is as toxic as the other for rabbits. These two strains differ then in two essentials: the virulent strain does not agglutinate within the circulation of the normal rabbit and is not opsonized by normal rabbit serum, while the non-virulent strain agglutinates instantly after injection into the circulation of normal rabbits and is opsonized by the serum of normal animals.

Miscellaneous Strains.—The following organisms have been found to agglutinate in the circulation of normal rabbits: *Staphylococcus aureus* and *albus*, *Bacillus coli*, meningococci, gonococci, and strains of *Bacillus mucosus capsulatus* that had been on artificial media for a number of months. None of the strains used in these experiments produced general infections in rabbits and injections of subtoxic quantities were without obvious effect.

Bacillus avisepticus in Dogs and Rabbits.—As is well known, *Bacillus avisepticus*, as well as other members of the hemorrhagic septicemia group, becomes exceedingly virulent for rabbits after a few passages in these animals. Indeed, rabbits often become infected with these organisms in nature. Dogs are, on the other hand, much more resistant to infection with organisms of this group and natural

infections are not known to occur. Hence in an effort to learn more of the mechanism of the natural resistance of animals to infection, it was deemed expedient to determine the fate and behavior of these organisms when injected into the circulation. A strain of *Bacillus avisepticus* was selected for this study.

Virulence and Toxicity.—It was soon found that cultures of *Bacillus avisepticus* are highly toxic for both rabbits and dogs, but that the results are quite different in the two species if subtoxic quantities of culture are given. This is illustrated in Table I, A and B.

TABLE I.

A.

Animal.	Weight.	Time.	Injection.*	Results.
	gm.		*cc.*	
Rabbit A.	1,700	10.40 a.m.	0.75	
		12.05 p.m.		Died. Intoxication.
" B.	1,800	10.40 a.m.	0.18	
		8.30 p.m.		Found dead. More bacilli than red cells in film from heart's blood.
" C.	1,700	10.40 a.m.	0.000005	Rabbit died 17 hrs. after injection. Heavy septicemia.

* 24 hour bouillon cultures were used and all injections were intravenous.

B.

Animal.	Weight.	Time.	Injection.	Results.
	kg.	*1915*	*cc.*	
Dog A.	9.0	Oct. 16, 10.15 a.m.	1.8	
		" 16, 11.15 a.m.		Prostrate; frothy vomitus.
		" 17, 9.00 a.m.		Sluggish; lying in cage.
		" 17, 3.00 p.m.		Died. 6 colonies from 2 loops of heart's blood.
Dog B.	14.75	Oct. 18, 10.45 a.m.	0.5	No symptoms of intoxication.
		" 19, 9.00 a.m.		0.1 cc. of blood put on agar slant; no colonies developed. Dog remained in good condition.

From Table I, A it is seen that bouillon cultures of *Bacillus avisepticus* are highly toxic for rabbits, 0.5 cc. of culture per kilo causing acute death. The intoxication is largely due to a toxin, since 1 cc. per kilo of body weight of a bacteria-free filtrate from a 24 hour culture causes acute death. Rabbits B and C show that a subtoxic dose of culture causes death within a few hours by a blood infection and that a very small quantity of culture is fatal. This organism seems to grow without restraint in the circulation of rabbits.

The experiments recorded in Table I, B show that a very small quantity of culture (0.2 cc. per kilo) causes a fatal intoxication in dogs, but that a subtoxic injection is without effect. This effect is quite different from that which occurred in the rabbits and proves that cultures of *Bacillus avisepticus* are exceedingly toxic for dogs but not infectious. What is the reason or mechanism of the resistance to the infection on the part of the dogs?

Bactericidal Action of Blood and Serum.—The serum and citrated and hirudinized blood of both dogs and rabbits were tested for their bactericidal action upon *Bacillus avisepticus*. The technique was as follows: 1 cc. of the fresh undiluted serum and blood was put in a series of tubes, and 0.05 cc. of 1:40,000 dilution of a 24 hour bouillon culture of the bacilli was added. Two salt solution controls were run. One control tube was plated immediately after it was set up. The other tubes were kept at 37°C. for 2 hours and then plated.

The fresh serum and whole blood of both dogs and rabbits proved to be good culture media for the bacilli. The incubated salt solution control was sterile, but in the blood and serum tests there was a tenfold increase over the control plated at the beginning of the experiment.

Opsonins.—The Neufeld method was followed in testing the opsonic power of the sera. The bacilli were incubated with the serum for an hour before the leukocytes were added. The results are given in Table II.

Guinea pig leukocytes were used in this experiment, and, as is shown in the table, rabbit serum failed almost entirely to opsonize the bacteria for these phagocytes. It was conceivable, however, that rabbit serum would opsonize the bacilli for other phagocytes, dog or rabbit. Hence other tests were made with serum-phagocyte

TABLE II.

Dilution.	Serum.	Degree of phagocytosis.
1:2	Dog.	++++
1:10	"	++++
1:20	"	++
1:40	"	+
1:2	Rabbit.	+
1:10	"	–

combinations as follows: rabbit serum + dog phagocytes, rabbit serum + rabbit phagocytes, and guinea pig serum + dog and rabbit phagocytes. The results of all these experiments proved conclusively that the explanation of the phagocytosis was the presence of the dog serum. Any of the leukocytes would phagocyte the bacilli actively in the presence of dog serum and none of them to any degree in the presence of the other sera (Figs. 1, 2, and 3).

Agglutinins.—Rabbit serum does not agglutinate *Bacillus avisepticus in vitro*, even in 90 per cent serum. In dog serum agglutination occurs only in the dilutions below 1:5. However, since it has been shown that bacteria agglutinate in the circulation of animals whose serum has little or no agglutinative power in the test-tube,[1] it was advisable to do the reactions *in vivo*.

Dog C.—Weight 9 kilos. The bacilli from four agar slants were suspended in salt solution and injected into the ear vein. Specimens of blood were taken from the heart, as indicated in Table III.

TABLE III.

Time after injection.	Clumps.	Free bacteria.
min.		
$\frac{1}{2}$	Few.	Many.
1	Many, large.	Few.
3	Some.	None.
5	None.	"

The dog was killed at the end of 30 minutes and portions of the lungs, liver, and spleen were crushed and examined for clumps. The lungs showed many large clumps and free bacilli; the liver, clumps

and a few free bacilli; the spleen, a few clumps. Leukocytes containing clumps of bacteria were found in the lung and liver specimens (Figs. 4 and 5).

The result of this experiment considered with those of other experiments indicates that these bacilli are agglutinated immediately upon entering the blood stream of dogs, and that the agglutinated bacteria accumulate in the lungs, liver, and spleen, where they are taken up by phagocytic cells.

Similar experiments were made with rabbits and it was found that the bacilli are not agglutinated, remain in the circulation, and soon begin to multiply (Fig. 6). Cultural methods showed that the injected bacilli gradually decrease in number for the 1st hour, at which time they begin to increase with marked rapidity.

DISCUSSION.

The results of the preceding experiments corroborate the occurrence and amplify the significance of the agglutination of bacteria within the circulation of infected or inoculated animals It has been pointed out that an inverse ratio seemed to exist between the power to produce septicemia and the degree of the agglutination of the infecting bacteria in the circulation of the host.[3] This conclusion was drawn from observations made with a few bacteria and with one animal species, the rabbit. The observations described above have a double import in that a large number of bacteria has been studied and the observations have been extended to other animals.

In the first place, it is shown that bacteria of low agglutinability in the test-tube are agglutinated with great facility in actively immunized animals. *Bacillus muscosus capsulatus* agglutinates with marked rapidity in the circulation of actively immunized rabbits, while the sera of the same animals do not agglutinate the bacilli in dilutions above 1:5 after several hours' incubation. Virulent pneumococci and Shiga dysentery bacilli are also readily agglutinated in the blood stream of immune rabbits. Hence it appears that active immunity insures the agglutination of the specific bacteria when they

[3] Bull, *J. Exp. Med.*, 1915, xxii, 489.

are injected into the circulation. It is possible, of course, that exceptions to this rule may be found.

The behavior of non-virulent pneumococci in the circulation of normal rabbits is especially interesting since it is so different from that of virulent strains. As far as was determined, these strains differed in only two respects: the septicemia-producing strains are neither opsonized nor agglutinated by normal rabbit serum, while the non-virulent strains are both agglutinated and opsonized, especially within the animal body. If these results are added to those obtained with such organisms as *Staphylococcus aureus* and *albus*, meningococci, gonococci, influenza bacilli, and *Bacillus coli*, the relation between agglutination and opsonization and the power to produce a general infection becomes evident; *i.e.*, they are inversely parallel.

This relation is again observed in comparing the infectiousness of *Bacillus avisepticus* for dogs and rabbits, and the fate of the bacilli when injected into the circulation of these animals. Here, also, the power of the serum to agglutinate and opsonize the bacteria was the only distinguishing feature. These results especially emphasize the significance of agglutinins and opsonins in the mechanism of natural resistance to infection, since *Bacillus avisepticus* produces a powerful toxin and is still incapable of causing a septicemia of any consequence in the presence of these antibodies.

It should be stated that we have no intention to urge that agglutinins and opsonins are all there is to the immunity forces, but we wish to emphasize the fact that they are operative within the circulation and organs of infected animals where they seem to play a decisive part in many instances.

SUMMARY.

1. Pneumococci, dysentery bacilli of the Shiga type, and *Bacillus mucosus capsulatus* are agglutinated immediately when injected into the circulation of actively immunized rabbits.

2. *Staphylococcus aureus* and *albus*, colon bacilli, meningococci, gonococci, and non-virulent pneumococci agglutinate in the circulation of normal rabbits.

3. Bouillon cultures of *Bacillus avisepticus* are highly toxic for both rabbits and dogs. The fresh sera of these animals have no bactericidal action upon the bacteria. Dog serum opsonized the bacilli *in vitro*, and they are agglutinated and opsonized in the circulation and organs of normal dogs. On the other hand, none of this occurs in connection with normal rabbits. A very small quantity of culture produces a fatal septicemia in rabbits, but a subtoxic dose is without effect in dogs.

4. The degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host.

EXPLANATION OF PLATE 7.

FIG. 1. *Bacillus avisepticus*, dog serum, and guinea pig leukocytes after 1 hour at 37°C.

FIG. 2. *Bacillus avisepticus*, dog serum, and rabbit leukocytes after 1 hour at 37°C.

FIG. 3. *Bacillus avisepticus*, rabbit serum, and guinea pig leukocytes after 1 hour at 37°C.

FIG. 4. A film from the heart's blood of a dog 1 minute after a suspension of *Bacillus avisepticus* had been injected into the ear vein.

FIG. 5. Phagocytosis of *Bacillus avisepticus* by a polymorphonuclear leukocyte. The preparation is from a dog's lung, the tissue being removed 30 minutes after the bacilli were injected.

FIG. 6. A film from the heart's blood of a rabbit 5 minutes after a suspension of *Bacillus avisepticus* had been injected.

34

THE AGGLUTINABILITY OF BLOOD AND AGAR STRAINS OF TYPHOID BACILLI.

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Gay and Claypole state that two transplantations on 10 per cent rabbit blood agar rendered typhoid bacilli non-agglutinable in an immune serum produced with plain agar cultures.[1] They further state that an immune serum obtained by means of blood agar cultures of the bacilli agglutinated equally well both blood and agar strains and that this serum agglutinated freshly isolated strains which were not agglutinated by an ordinary immune serum, thus rendering an early identification of the strains possible. They hold also that the inagglutinability of recently isolated strains is not due to some property acquired in the living animal, as a simple subculture for two generations on blood agar produces similar effects.

With a view of producing a general agglutinating serum according to this method, we first attempted to render our laboratory strains inagglutinable by growing them on 10 per cent rabbit blood agar. Two generations on blood agar failed to change the agglutinability of these strains. Cultivation on blood was continued and the agglutinability of the strains was tested after each two or three transfers. A detectable difference between the blood and agar strains did not arise, even after twenty-five generations. Since it was possible that our results were due to the particular culture with which we were working, fifty-five other cultures have been collected and subjected to a similar study.

The Strains.

We are indebted to Dr. Homer F. Swift of the Presbyterian Hospital, Dr. F. B. Humphreys of the German Hospital, and Dr. L. M.

[1] Gay, F. P., and Claypole, E. J., *Arch. Int. Med.*, 1913, xii, 621.

Famulener of St. Luke's Hospital for a number of the strains used in this work. We are especially indebted to Dr. Famulener for sending us twenty strains as they were isolated from patients. There were in all twenty-five freshly isolated strains. These were transferred to blood agar as soon as they had been identified. The remainder of the strains had been under artificial cultivation for a number of months.

Technique.

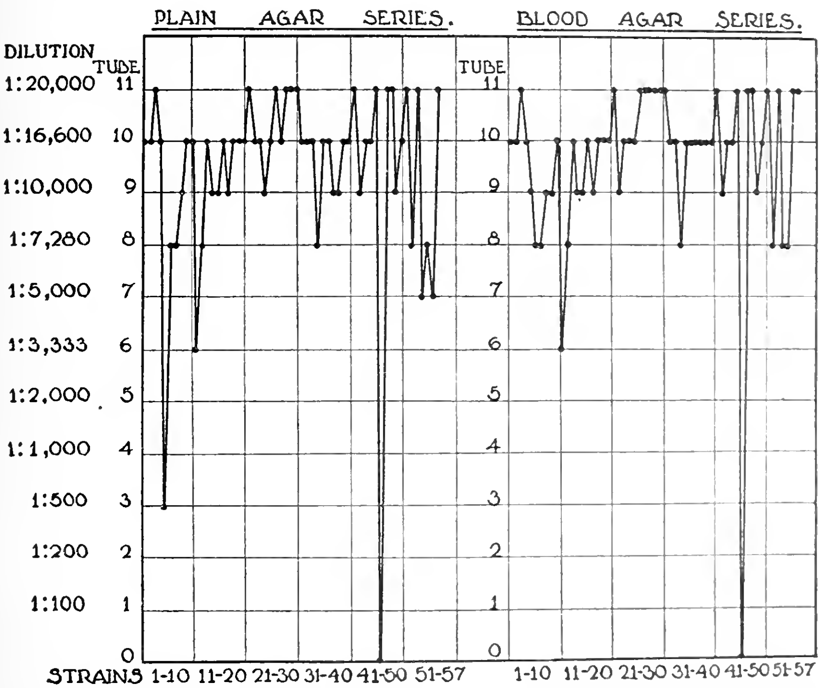
The serum used to test the agglutinability of the various strains was obtained by immunizing rabbits with a typical typhoid bacillus grown on plain agar. It agglutinated the homologous strain in a dilution of 1:20,000. The following procedure was used in making all the tests.

Graded dilutions of the serum were made with physiological salt solution. The dilutions ranged from 1:100 to 1:20,000. 1 cc. of the different dilutions was transferred to small test-tubes and one drop of bacillary emulsion was added to each, and also to a salt solution control. The emulsions of the bacilli were made by adding from 4 to 5 cc. of salt solution to 24 hour cultures on blood agar and plain agar slants, respectively. The blood and agar cultures of each strain were always tested on the same day and with the same serum dilutions. A special effort was made to have the emulsions from the corresponding blood and agar cultures of the same thickness. The tests were incubated for 2 hours at 37° C. and allowed to stand at room temperature for 2 hours before the results were read. A second reading was made the following morning. There was no difference in corresponding blood and agar cultures at the two readings. The controls were never agglutinated.

Results of the Agglutination Tests.

Fifty-seven strains were grown on blood agar for twenty-five generations and tested for agglutinability at varied intervals according to the technique just described. Only slight differences between the corresponding blood and agar strains were detected. The blood cultures were often more agglutinable than the agar cultures. There

was, however, considerable variation of the individual strains (both blood and agar cultures), and one inagglutinable strain was encountered. This strain was irregular in other particulars and will be considered more in detail below. The results are presented graphically in Text-figs. 1 and 2.

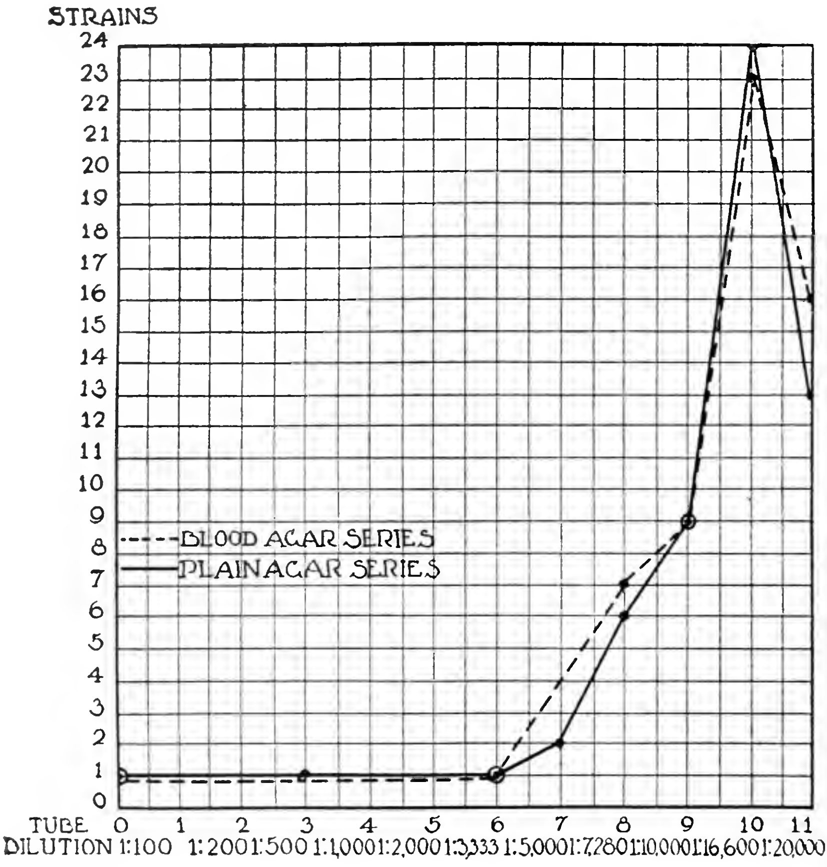


TEXT-FIG. 1. This figure shows the individual variations among the fifty-seven different strains. The dots on the abscissæ represent the individual strains from 1 to 57, in the order in which they were tested. The highest dilutions in which the strains agglutinated are represented by the ordinates.

The Irregular Strain.

This strain was brought to us by Dr. Amoss from the laboratory of Hygiene, Vermont State Board of Health. It had been recently isolated from the stool of a clinical case of typhoid and was typical according to the cultural methods ordinarily used to identify typhoid bacilli. We had not observed any irregularities in the strain until

it was found to be non-agglutinable with the immune serum used in the routine agglutination tests already described.



TEXT-FIG. 2. In this figure composite curves of the agglutinability of the blood and agar strains are given. The dilutions are represented by the abscissæ and the number of strains agglutinating in the different dilutions is represented by the ordinates.

Agglutinability.—In the routine tests the Vermont strain failed to agglutinate in a 1:100 dilution of the serum that agglutinated a number of other strains in a 1:20,000 dilution. It was later found that a dilution containing 50 per cent serum would not agglutinate

either the blood or agar cultures of this strain. It was agglutinable by fresh normal rabbit serum in a dilution of 1:5. It agglutinated in the circulation of normal rabbits as readily as other strains. A homologous serum produced by immunizing a rabbit with agar cultures agglutinated both the agar and blood cultures actively. This serum agglutinated typical strains of bacilli but not in as high dilutions as the homologous strains.

It is of interest to note the differences exhibited by the natural and acquired agglutinins. An immune serum that had stood on ice for several days failed to agglutinate the Vermont strain in 50 per cent serum, while normal rabbit serum agglutinated it in a 1:5 dilution. This indicates a lack of specificity on the part of natural agglutinins and shows further that natural agglutinins disappear spontaneously as the serum ages. The same normal serum agglutinated other strains as high as 1:20, hence the Vermont strain was naturally of lower agglutinability.

Cultural Characteristics.—In Hiss' carbohydrate serum-water media the following reactions occurred: Dextrose gave acid, no gas; levulose, acid, no gas; dextrin, acid, no gas; mannite, maltose, galactose, lactose, and saccharose gave no acid, no gas. Litmus milk gave slight acidity. Gelatin was not liquefied. Growth on potato media was typical. It produced indol as readily and as abundantly as *Bacillus coli*.

It is seen that the fermentation reactions of the Vermont strain are irregular, no acid being produced in galactose, mannite, or maltose. Control tests were run with other strains and typical reactions occurred in all the media.

The most radical discrepancy is the production of indol. This reaction has been accepted as a highly dependable test in differentiating typhoid bacilli from other closely related organisms ever since it was first used for this purpose by Kitasato.[2] Andrejew claims that a number of strains with which he worked produced indol.[3] Telle and Huber have more recently tested a number of strains and they obtained only negative results.[4] These authors believe that

[2] Kitasato, S., *Z. Hyg.*, 1889, vii, 515.

[3] Andrejew, P., *Arb. k. Gsndhtsamte.*, 1910, xxxiii, 363.

[4] Telle, H., and Huber, E., *Centr. Bakteriol., 1st Abt., Orig.*, 1911, lviii, 70.

Andrejew's results were due to faulty technique. All text-books on bacteriology teach that typhoid bacilli seldom or never produce even a trace of indol when tested by a standard technique. Therefore, the production of indol by supposed typhoid bacilli must be considered as a radical irregularity and their identity as seriously questioned.

Protection Tests.—Rabbits that have been immunized with typhoid bacilli are highly resistant to intoxication with this organism. They withstand, as a rule, from thirty to forty lethal doses of the living bacilli. The Vermont strain was highly toxic for rabbits and we decided, therefore, to test the resistance of rabbits which had been immunized with a typical strain for this strain. It was found that the rabbits would withstand from fifty to seventy-five lethal doses of the irregular strain.

The resistance of the typhoid immune rabbits to intoxication by the irregular strain either proves the typhoidal nature of the strain or indicates a marked non-specificity of the toxic substances derived from such organisms. However, the protection tests combined with cross-agglutination seem to establish the Vermont strain as a true typhoid bacillus.

SUMMARY.

Cultivation on 10 per cent rabbit blood agar did not affect the agglutinability of fifty-seven strains of typhoid bacilli.

The authors were unable to confirm the observations of Gay and Claypole on the variation in agglutinability caused by cultivating the typhoid bacillus on blood agar.

A typhoid bacillus showing irregularity in fermentation, agglutination, and indol production is described.

A FINAL REPORT ON THE CULTIVATION OF THE TUBERCLE BACILLUS FROM THE SPUTUM BY THE METHOD OF PETROFF.

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The purpose of this paper is to present a final report on the isolation of the tubercle bacillus from the sputum according to the method of Petroff.[1] From my early experience[2] it seemed to me that the method offered many opportunities, but my success at first was not great. In the present work an attempt has been made to control the possibilities which might influence a favorable or an unfavorable result. In a later communication Petroff[3] reports having isolated and cultivated the tubercle bacillus from 129 sputa out of 135 specimens. I have not been able to obtain this positive percentage, but I believe that after a little preliminary experience the results should be nearly as good.

Those accustomed to working with the tubercle bacillus realize that the results are variable, and that a method which will simplify or advance the present knowledge of tuberculosis will be of value. The method as devised by Petroff seems to open up many avenues of study in relation to the clinical course of tuberculosis and the tubercle bacillus.

According to my experience there are two important points to be realized. First, the demonstration of the tubercle bacillus unde-

[1] Petroff, S. A., A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces, *J. Exp. Med.*, 1915, xxi, 38.

[2] Keilty, R. A., A Study of the Cultivation of the Tubercle Bacillus Directly from the Sputum by the Method of Petroff, *J. Exp. Med.*, 1915, xxii, 612.

[3] Petroff, S. A., Some Cultural Studies on the Tubercle Bacillus, *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 276.

monstrable in sputum by other means. By microscopic examination of the cultures this is possible even though they become contaminated later by a diffuse growth of organisms. Second, the isolation of the tubercle bacillus and its study in pure culture.

The method of Petroff briefly is as follows: About 5 cc. of fresh sputum in a sterile bottle are mixed with sterile 3 per cent sodium hydroxide solution and incubated at 37°C. for 30 minutes. This should be frequently shaken and the mucin thoroughly broken up. A longer period in the incubator may be necessary for this. Place a piece of sterile litmus paper in the bottle and neutralize with sterile normal hydrochloric acid, centrifugalize at high speed for 10 minutes, and plant the sediment on three tubes of the gentian violet egg veal medium. Incubate as nearly as possible between 38° and 39°C. Variations in temperature will retard the growth decidedly.

Preparation of the Medium.—Many different modifications of egg media have been tried in the laboratory, and I find that where the whole egg is used there is not much difference. Possibly where starch is added the luxuriance of the growth is increased. From this I have lately used the medium of Petroff exclusively with some modification in the sterilization.

Meat.—500 gm. of fresh lean veal are finely ground up and infused cold over night in 500 cc. of a 15 per cent solution of glycerin in water. Squeeze out either by press or by twisting in gauze, and filter.

Eggs.—Break one dozen eggs into a large vessel and beat thoroughly. Filter through gauze. Add one part by volume of meat juice to two parts of egg.

Gentian Violet.—Add sufficient 1 per cent alcoholic solution of gentian violet to make a dilution of 1 : 10,000 to one-half of the medium and tube. Tube the other half without the gentian violet.

Sterilization.—Petroff advised the sterilization of all vessels, presses, and eggs before breaking, as recommended for Dorcet's medium. It was found difficult to prevent breaks in technique, and later experience proved that absolute sterility is unnecessary.

Make up the medium in the morning after the infusion of the veal over night. Tube from 3 to 5 cc. and pack in the inspissator at about 1 p.m. Bring the temperature up to 95°C. as quickly as possible, avoiding bubbles, and keep it at this point for 1 hour. Temperatures over 100°C. will spoil the medium. Repeat the process in the inspissator at 95°C. on 2 successive days. In the early work there was considerable trouble with contamination of the medium, but with the technique carefully watched these troubles have been entirely eliminated. This should give about 125 tubes, one-half with and one-half without gentian violet. Considerable water of condensation will result. This is allowed to evaporate for a few days without dryness and the tubes are sealed as follows:

Paraffin Mixture.—Seven parts of paraffin at 55°C., one part of yellow bee's wax, and one part of petrolatum. Heat over the Bunsen as hot as possible with-

out combustion. Flame the top of the tube and cotton plug, remove the latter with sterile forceps and flame it. Immerse while burning into the hot paraffin mixture and plug, allowing the excess to drain back into the mixture. Heat the paraffin after every dozen tubes. We have never had an infection by using this method. When the tubes are in use heat before and after removing the plug. In this way tubes may be kept for as long as 8 months and frequently opened without much drying of the medium or contamination. I have had an opportunity of observing cultures obtained from many different laboratories, and find that the technique of sealing varies considerably and in some cases at least is impracticable for frequent use of the tube. For this reason and because drying was one of my earliest difficulties the method has been included in some detail.

Results.—Twenty-eight consecutive specimens were studied, most of which were kindly furnished by Dr. Baldwin Leuke, from the wards of the Philadelphia General Hospital. These were not selected in any way. They were collected during the night in sterile bottles and started the next day. The fact that they were not absolutely fresh has no doubt influenced the result somewhat. Fresh sputum as suggested by Petroff is important. In 2 of the 28 cases the tubes were broken and lost in the centrifuge and 1 was too old so that 25 cases were finally studied. Of the 25 cases, 23 were said to be advanced, 1 case, a student, undoubtedly negative, and 1 case suspected but probably negative. On examination by smear, 18 cases were positive and 7 negative for tubercle bacilli after 5 minutes' examination. Of the 18 positive cases, 12 showed cultural evidences of growth, and of the 7 negative cases, 1 showed growth. Of the 12 positive cases, 4 were obtained in pure culture and 8 showed microscopic evidence of growth; that is, colonies were too minute to be made out grossly or were overgrown by contamination, but upon microscopic examination showed groupings in numbers sufficient to exclude any doubt that they were carried over from the sputum originally. Where any doubt existed they were classed as negative. Of the 4 pure cultures, 2 averaged 10 to 14 organisms to a field upon the original sputum examination. 2 showed 1 to several fields. Of the 18 positive sputa, only 4 had many organisms to a field and 2 of these gave pure cultures, while 1 had microscopic evidence in culture and 1 had to be incubated 24 hours because of a thick ropy sputum. Of the 4 pure cultures, 1 case showed 2 contaminations out of 4 tubes inoculated. The other 3 cases grew with-

out contamination. Case 20, a negative case, was several days old when the specimen was received. 3 cases showed early microscopic evidence of growth, 1 on the 3rd day and 2 on the 4th day. This number probably would have been increased if the tubes had been examined more frequently. The first appearance of gross growth averaged 14 to 21 days. This result was dependent upon opportunities for observation and in some cases the time may be shorter. In transplants from young cultures I have frequently observed growth in 3 or 4 days and good heavy growth in 10 days on the plain egg medium.

The colonies first visible under a magnifying glass are pin-point. They dot up like minute mushrooms, tending to heap up; if scattered they will grow with umbilicated centers. They spread over one another with a typical dry hilly pattern if close together. When pure growth is first determined they should be immediately transferred to plain egg medium upon which they grow luxuriantly. They often fill the entire surface of the medium at the end of a month when they should again be transplanted. There seems to be a great variation in the rapidity of growth of different cultures. This depends upon the culture, the medium, and the incubation. The medium must be made and sterilized correctly. One batch, upon which no growth took place, was ruined by oversterilization. This is, however, an old experience with egg media. The moisture in the tube has a great deal to do with growth. The cultures will not thrive with visible water of condensation; on the other hand, they will not grow on a dry glazed surface. Finally there are unexplainable reasons for failures to grow. One series, No. 380, a subculture, was planted on ten tubes of plain egg on Jan. 23, 1916. The following note was made on Feb. 22: five tubes grew profusely, three scantily, and two were negative. All were subjected to the same conditions. For this work, therefore, it is advisable to use at least three tubes.

Contamination.—In the original planting three tubes of gentian violet medium were used. Where contaminations occurred they showed up, as a rule, at the end of 24 hours as small colonies, as diffuse, moist, smeary growths, or as localized colonies. In the majority of cases this contamination seemed to be held in check thereafter by the gentian violet; while in others the organisms continued

to grow, and in a few instances completely liquefied the medium. The contaminated cultures should be watched carefully and where growth is checked it may be possible to pick out a single colony of tubercle bacilli, transplant it, and thus obtain a pure culture. Where this is not possible, at least evidence of microscopical growth may be obtained.

Brief History of the Pure Cultures.

No. 326.—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes at high speed.

Dec. 22. Two tubes are contaminated and discarded.

Dec. 23. A third tube shows microscopic growth with small white colonies contaminating, a large coccus. A small bit of uncontaminated material transferred to a plain egg tube No. 326 C.

Jan. 23, 1916. No. 326 C has a scattered growth in raised, dry colonies.

Feb. 5. A profuse dry growth of acid-fast bacilli. Transplanted.

Mar. 15. Culture growing profusely.

No. 328.—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes.

Dec. 22. No growth.

Jan. 4, 1916. Shows the first evidence of growth, one small colony of pure acid-fast bacilli. Transferred to three tubes of plain egg medium, Nos. 328 A, B, and C.

Jan. 23. No. 328 C is growing well as multiple, whitish colonies tending to spread. Original tube did not continue to grow.

Mar. 15. Culture continues to grow after transplanting.

No. 364.—Jan. 6, 1916. Sputum incubated for 6 hours; slightly acid in reaction; centrifugalized 30 minutes at high speed.

Feb. 9. Two or three minute colonies the color of the medium; acid-fast bacilli in pure culture. Transplanted to plain egg medium, Nos. 364 A, B, and C.

Mar. 1. Has grown into dry, raised, granular colonies not tending to spread but to heap up.

No. 365.—Jan. 6, 1916. Sputum incubated 6 hours; slightly acid in reaction; centrifugalized for 30 minutes.

Feb. 9. Small raised colonies the color of the medium. Transplanted to plain egg medium, Nos. 365 A, B, and C.

Mar. 1. Original gentian violet tube and No. 365 C growing profusely. Nos. 365 A and B did not grow.

Swabs.—In twelve cases, Nos. 1 to 9 and 13 to 15 inclusive, sterile cotton swabs were thoroughly rubbed over the tonsils, fauces, tongue, and gums. The swabs were replaced in tubes and the latter

filled with 3 per cent sodium hydroxide. They were then incubated for 30 minutes. The sodium hydroxide was poured off except enough to cover the cotton swab. This was neutralized and the fluid centrifugalized. Both the sediment and the cotton removed from the applicator were planted on gentian violet egg tubes. The purpose in this portion of the work, the report of which is merely preliminary, is to obtain a method for the demonstration of the tubercle bacillus where sputum is not obtainable; for example, in children and in the insane.

In one instance, No. 371 from Case 14, the preliminary smear being negative, a pure culture was obtained. The history of this culture is as follows: The swab was obtained on Jan. 8, 1916; the smear from this was negative. On Jan. 17, one minute colony with pure acid-fast bacilli was present. It was transplanted to plain egg medium No. 371 X. On Mar. 15, No. 371 X has a good profuse growth of acid-fast bacilli in pure culture. The specimen of sputum from this case showed microscopic evidence of growth in 72 hours, but pure culture was not obtained owing to contamination.

This is a preliminary report as regards the swab, but it has possibilities with improved technique which will be reported on later. It may therefore be concluded that it is possible to isolate the tubercle bacillus in pure culture by Petroff's method from a swab of the throat and mouth.

As a control on the sputum work Dr. J. D. Paul of the resident staff of the Episcopal Hospital of Philadelphia examined some sputa in the laboratory of that Hospital. He selected ten cases from the dispensary service and I am indebted to him and to the Director of the Laboratory, Dr. C. Y. White, for the following report. The work was carried on entirely independently of me. From the ten cases four cases in pure culture were recovered. This gives a higher percentage of positive cases than my own work. In addition he recovered a pure culture by applying the technique to a specimen of pus from a tuberculous knee.

The method has not been studied in regard to feces, and the results with tissue have been negative as far as obtaining growth is concerned. In regard to tissue, small pieces are completely digested in the sodium hydroxide after incubation for 24 hours. From the sediment after centrifugalization the tubercle bacillus can be demonstrated

even when it is impossible or requires considerable time to demonstrate it in a section. From this result a 3 per cent sodium hydroxide solution is as good as antiformin for this purpose.

TABLE I.

Summary of Results.

No. of specimen.	No. of culture.	Date.	Type of case.	Smear.	Organisms per field.	Tubes planted.	Contaminations in 24 hrs.	Contaminations in 1 wk.	First growth.	Type of result.	Result.
		1915							*1916*		
1	326	Dec. 20	Adv.*	+	14	4	2	0	Jan. 23	Pure.	⊕
2	327	" 20	"	—	0	3	0	0			—
3	328	" 20	"	+	10	3	0	0	" 4	"	⊕
									1915		
4	336	" 22	"	+	Occ.	3	1	0	Dec. 26	Micro.	+
5	337	" 22	"	+	"	3	1	0	" 26	"	+
6	338	" 22	"	+	"	3	3	0			—
		1916									
7	355	Jan. 4									
8	356	" 4									
9	357	" 4	"	—	0	3	1	0			—
									1916		
10	363	" 6	"	+	Occ.	3	0	3	Feb. 8	"	+
11	364	" 6	"	+	1	3	0	0	" 8	Pure.	⊕
12	365	" 6	"	+	Occ.	3	0	0	" 8	"	⊕
13	367	" 8	"	+	"	3	0	0	" 9	Micro.	+
14	368	" 8	"	+	"	3	2	0	Jan. 11	"	+
15	369	" 8	"	—	0	3	1	3			—
16	375	" 16	?	—	0	3	3	0			—
17	376	" 17									
18	377	" 17	Adv.	+	15	3	0	0			—
19	378	" 17	"	+	15	3	3	0	Feb. 16	"	+
20	401	Feb. 23	?	—	0	5	5	0			—
21	412	" 27	Adv.	+	Occ.	3	1	3			—
22	413	" 27	"	—	0	3	3	0			—
23	414	" 27	"	—	0	3	0	0	Mar. 23	"	+
24	415	" 27	"	+	Occ.	3	0	0	" 23	"	+
25	426	Mar. 3	"	+	"	3	0	0			—
26	427	" 3	"	+	4	3	2	0	" 23	"	+
27	428	" 3	"	+	4	3	0	0			—
28	431	" 4	"	+	Occ.	3	0	0			—

* Adv. indicates advanced; ?, suspected; Occ., occasional; Micro., microscopic evidence of growth; ⊕, pure culture.

DISCUSSION AND SUMMARY.

In summing up the practicability of the Petroff method which is in reality a refinement and improvement upon well known methods, it has possibilities both from the standpoint of diagnosis and the study of the tubercle bacillus. It is not a difficult technique, but requires considerable attention to detail. The results should be better in larger series of cases carried on in laboratories where more time is available for close observation of the cultures during the early periods of development. It is the early transplanting of minute colonies even in the presence of contaminations which results in pure cultures.

As to the practical value of the method, my experience would lead me to conclude that where sputum is obtainable in suspected cases of tuberculosis in which the tubercle bacillus cannot be demonstrated, an opportunity for diagnosis would be missed if this technique were not applied. If the result is negative it means nothing; if positive an otherwise obscure case may be cleared up. The Petroff method offers an easy opportunity of isolating large numbers of pure cultures (Table I).

CONCLUSIONS.

1. The method of Petroff offers an opportunity for the isolation of the tubercle bacillus in pure culture from sputum.
2. The method is available for the demonstration of the tubercle bacillus from sputum when otherwise not demonstrable.
3. The method is easy and rapid, but requires detail and constant observation.
4. The plain egg veal medium is the best medium thus far devised for the luxuriant growth of the tubercle bacillus.
5. The method supersedes antiformin, having all its good qualities and others in addition.
6. It is possible by applying the method to isolate the tubercle bacillus from a swab of the throat.
7. By using the digestive powers of sodium hydroxide it is possible to demonstrate more readily the presence of the tubercle bacillus in tissue, thus saving time on animal inoculation.
8. Finally, if positive results are obtained from an otherwise negative case the method opens up one more avenue of diagnosis.

VARIATIONS IN THE PNEUMOCOCCUS INDUCED BY GROWTH IN IMMUNE SERUM.

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The change produced in bacteria by growth in specific immune sera has been studied for many years. Metchnikoff (1) in 1887 found that the virulence of anthrax bacillus seemed to be diminished by growth in anti-anthrax serum. Similar results were obtained by Charrin and Roger (2) and Roger (3) with *B. pyocyaneus*, pneumococcus, and streptococcus. Later investigations, however, showed that this apparent loss of virulence was due to the protective action of the immune serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in their virulence (Metchnikoff (4), Sanarelli (5), Issaeff (6)). The study of the effect of this treatment has been confined chiefly to observations on *B. choleræ*, *B. typhosus*, and other intestinal organisms (von Ransom and Kitashima (7), Müller (8), Eisenberg (9), Walker (10), Smith and Reagh (11), and Feiler (12)). Investigators have found that bacteria grown in homologous immune sera, or in the peritoneal cavities of immune animals, show marked increase in virulence and in resistance to bactericidal action, and a loss of agglutinability. The increase in virulence and in resistance to bactericidal action would seem to indicate a biological adaptation on the part of the bacteria, similar to the process of increased resistance which the animal organism develops when invaded by infectious agents. The loss of agglutinability of the organisms treated with immune serum has been explained as being possibly attributable to an inadequate receptor apparatus, this condition having been brought about by the injurious action of the immune serum upon the organism. Joos (13) has demonstrated that such an effect is brought about by heating typhoid bacilli. He finds that the agglutinin-producing substance, agglutinogen, of typhoid bacilli apparently consists of two elements, which he designates as α- and β-agglutinogen. The α-agglutinogen is easily destroyed by heating at 60–62°C., while the β-agglutinogen is heat-resistant. Injection of living unheated bacilli produces both α- and β-agglutinins, while the injection of heated bacilli produces only β-agglutinins. Cole (14) has found that inagglutinable typhoid strains possess less power to absorb agglutinins than do normal strains, and, when injected into animals, produce sera which are not as highly agglutinating, even for the injected strain, as the sera produced by highly agglutinable strains. These experiments seem to show that the receptor apparatus of the inagglutinable strains is not so complete as that of the agglutinable bacilli.

Recently Friel (15) has found that by growing pneumococci in immune serum they became agglutinable and phagocytable in normal rabbit serum, and less virulent for mice than the untreated strains.

The present investigation was undertaken in order to study the different types of pneumococcus with regard to this phenomenon and to determine whether any variation of type organisms occurs after treatment with Antipneumococcus Serum I or II.

The experiments were made with virulent strains of the pneumococcus, freshly isolated from the heart's blood of mice dead from pneumococcus septicemia, or obtained from blood cultures from cases of lobar pneumonia. In all experiments determinations of morphology, bile solubility, inulin fermentation, capsule formation, virulence, and agglutination were made before the strain was subjected to treatment. Cultures were then made in normal and immune serum bouillon, and, in some instances, as a control, in plain bouillon. All serum media were prepared by the addition of 0.5 cc. of serum to 4.5 cc. of the nutrient beef infusion bouillon used for routine culture medium, the immune serum bouillon containing one part in ten of a highly potent antipneumococcus horse serum, the normal serum bouillon containing the same amount of normal horse serum. Cultures were made by inoculation of 0.5 cc. of a bouillon culture of the strain to be tested, the tubes were then incubated at 37°C. for 18 hours, and after that time 0.5 cc. of this serum culture was reinoculated directly into another tube of serum bouillon. In some instances cultures were transferred daily, in others at weekly intervals, being kept in the refrigerator during the intervals between incubation. Two different series of subcultures of each strain tested were employed, one being cultured consecutively in immune serum bouillon, the other in normal serum bouillon. The manner of growth and morphology of the organisms were noted each time that a culture was transferred. It was noted that all strains grew luxuriantly under the conditions and could be kept growing, by repeated transfer, for indefinite lengths of time. Two strains have now been grown under such conditions for over a year. Type strains grown in the homologous immune serum showed at first complete agglutination, the growth sedimenting after 18 hours' incubation in a hard mass which could be broken apart only with difficulty. After five or six transfers of Type

I in homologous serum, the growth sedimented in a flocculent mass which could be easily shaken apart. In order to produce the same effect with Type II organisms, twelve to fifteen transfers in immune serum were necessary. The microscopic picture was that of the well known thread reaction, individual organisms presenting a swollen appearance and growing in long chains or clump formation. All strains retained the original characteristics of bile solubility, inulin fermentation, and reaction to Gram stain. Marked differences, however, between the immune and normal serum treated organisms were noted in growth on blood media, agglutination, capsule formation, pathogenicity for white mice and rabbits, and antigenic and opsonic reactions.

Type strains which had been grown for a number of transfers in immune serum lost the characteristic moist, confluent, greenish growth on blood media and grew in dry, isolated, brownish colonies which showed a tendency to produce hemolysis of the blood cells in the media. When grown in plain bouillon subsequent to serum treatment, these strains showed marked sedimentation, this characteristic persisting even after twenty-five passages in plain bouillon. They also showed less tendency to form methemoglobin than the normal serum treated strains.

Variations in Agglutination.

The change noted was the development of a non-specific agglutinative reaction by the immune serum treated strains. Those strains which previously were agglutinable only in the homologous serum, subsequently were agglutinated somewhat less completely in this serum, and were also agglutinated by heterologous immune sera, and in some instances even by normal serum. All agglutination tests were made according to the following routine. 0.5 cc. of the 1:10 serum culture was inoculated into 5 cc of plain bouillon; after this subculture had grown from 4 to 6 hours, 0.5 cc. was inoculated into 25 cc. of plain bouillon, and this second subculture incubated for 18 to 20 hours. At the end of that time the bacteria were centrifugalized out, washed twice in saline, and an emulsion of these washed organisms in salt solution was used for the agglutination test. In this way, the error that would result from the pres-

ence of the immune serum in which the organisms had been grown was avoided. It will be seen that the last subculture contained serum in dilution of only 1:5,000; after washing twice in saline even this small amount must have been largely removed. Antipneumococcus serum does not agglutinate in a dilution of immune serum higher than 1:500, hence the reaction obtained could not have been due to a small amount of serum adhering to the bacteria. Agglutinations were made with the two types of antipneumococcus horse serum, and with normal horse serum. In each case one tube contained equal parts of the undiluted serum and bacterial emulsion, and the others 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Tubes were kept in a water bath at 37°C. for 2 hours, and over night on ice, and readings were taken at the end of that time. Complete reaction (++) was recorded when the bacteria were entirely sedimented with marked precipitation, leaving the supernatant fluid clear; almost complete (+±), when the bacteria were sedimented with less marked precipitation; incomplete (+), when marked clumping could be seen but the clumps were still readily shaken from the bottom of the tube; partial (±), when the clumps had not sedimented, but could be distinguished macroscopically as a fine granulation; negative (−), when no clumping was present.

Table I shows the result of three agglutination tests, the organism in each instance having descended from the same mouse passage culture of Pneumococcus Type I. The original culture showed typical agglutination reaction, virulence, and protection by homologous serum. Subculture A was grown successively in Immune Serum I bouillon, Subculture B successively in normal serum bouillon, Subculture C successively in plain bouillon. After 4 immune serum treatments, Subculture A lost intensity of reaction with its homologous serum, from complete to incomplete, and became partially agglutinated with the heterologous serum. After 20 treatments in immune serum the reaction was still incomplete with the homologous serum and had become almost complete with the heterologous serum. After 15 transfers in plain bouillon, subsequent to 22 treatments in immune serum, the organisms had regained somewhat in intensity of reaction with the homologous serum, but still agglutinated partially with the heterologous serum. Subcultures B and C, on the contrary, maintained

TABLE I.

Agglutination Tests of Serum Treated Strain I 114, Pneumococcus Type I, 114 Animal Passages, before Serum Treatment.

Subcultures in each test were grown for two successive transfers in plain bouillon subsequent to serum treatment and a twice washed emulsion of centrifugalized organisms was used. Tube 1 in each case contained 0.3 cc. of bacterial emulsion and 0.3 cc. of undiluted serum; the remaining tubes contained 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Readings were made after the tubes had been kept in a water bath at 37°C. for 2 hours and over night on ice.

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
	Strain I 114^{7}*. 4 serum treatments.								
Nov. 6, 1915.									
Undiluted.	+	±	−	++	−	−	+	−	−
1:10	+	±	−	++	−	−	+	−	−
1:20	+	±	−	++	−	−	++	−	−
1:40	+	−	−	++	−	−	++	−	−
1:50	+	−	−	+	−	−	++	−	−
1:100	+			−			−		
1:200	±			−			−		
1:400	±			−			−		
1:500	−			−			−		
Salt control.	−			−			−		
	Strain I 114^{23}. 20 serum treatments.								
Nov. 30, 1915.									
Undiluted.	+	+±	−	++	−	−	++	−	−
1:10	+	+±	−	++	−	−	++	−	−
1:20	+	+±	−	++	−	−	++	−	−
1:40	+	+±	−	++	−	−	++	−	−
1:50	+	+±	−	++	−	−	++	−	−
1:100	+			−					
1:200	+			−					
1:400	+			−					
1:500	+			−					
Salt control.	−			−					

TABLE I—*Concluded.*

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain I 114^{38}. Dec. 21, 1915.	15 transfers in plain bouillon subsequent to serum treatment.								
Undiluted.	±	±	−	+	−	−			
1:10	++	±	−	+	−	−			
1:20	+	±	−	+	−	−			
1:40	+	±	−	+	−	−			
1:50	+	±	−	+	−	−			
1:100	+			+					
1:200	+			−					
1:400	−			−					
1:500	−			−					
Salt control.	−			−					

* In all the tables the numerals after the culture indicate the number of mouse passages. The exponent indicates the number of generations removed from the last passage.

throughout the course of treatment the same intensity of reaction with the homologous serum and did not become agglutinated either in the heterologous or in the normal serum.

Table II shows the result of three agglutination tests made with two subcultures, both from the same mouse passage culture of a Pneumococcus Type II, which showed typical agglutination reaction, virulence, and protection before treatment. Subculture A was grown successively in Immune Serum II bouillon, Subculture B successively in normal serum bouillon. After 53 treatments with immune serum, Subculture A was only partially agglutinated in the homologous Serum II, but was completely agglutinated in the heterologous Serum I, and incompletely agglutinated in normal serum. After 75 treatments in immune serum, it was still only partially agglutinated in the homologous serum, completely agglutinated in the heterologous, and, this time, not agglutinated in normal serum. After 47 transfers in plain bouillon subsequent to serum treatment, the partial agglutination with the homologous serum and complete agglutination with the

TABLE II.

Agglutination Tests of Serum Treated Strain II 34, Pneumococcus Type II, 34 Animal Passages, before Serum Treatment.

The same technique was used as in the tests in Table I.

Serum dilutions.	Subculture A treated in Immune Serum II.			Subculture B treated in normal serum.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain II 34^{68}. 53 serum treatments. Oct. 1, 1915.						
Undiluted.	++	±	+	−	++	−
1:10	++	±	+	−	++	−
1:20	++	±	+	−	++	−
1:40	+	±	+	−	++	−
1:50	+	±	+	−	++	−
1:100		±			++	
1:200		±			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	
Strain II 34^{90}. 75 serum treatments. Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	++	±	−	−	++	−
1:50	++	±	−	−	±	−
1:100	++	±			−	
1:200	−	−			−	
1 400		−			−	
1:500		−			−	
Salt control.		−			−	
Strain II 34^{116}. 47 transfers in plain bouillon subsequent to serum treatment. Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	+	±	−	−	++	−
1:50	+	±	−	−	++	−
1:100		±			−	
1:200		−			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	

heterologous serum persisted. In this experiment, also, the organisms grown in normal serum were still completely agglutinated with homologous serum and were in no instance agglutinated with the heterologous or normal serum. It is to be noted, however, in spite of the incompleteness of the agglutination of the treated organisms in the homologous serum, that the agglutination titer in homologous serum was higher than that in heterologous serum, and also higher than that of normal untreated strains in homologous serum.

These tests were repeated a number of times with many different strains, always with the same result; the longer a strain was treated with its homologous serum, the less agglutinable it became in that serum, and the more agglutinable in the heterologous serum. On the contrary, the control strains cultured in normal serum and plain bouillon, retained throughout the experiments the typical agglutination with the homologous serum and were never agglutinated by the heterologous or normal serum. It was also noted that the immune serum treated strains sometimes were agglutinated with normal horse serum, but they did not agglutinate spontaneously in salt solution, nor did they agglutinate in other immune sera, such as diphtheria and tetanus antitoxin, anti-influenza serum, nor in an immune sheep serum obtained by the injection of an antigen prepared by the solution of pneumococci in sodium cholate. Bull (16) has tested an immune serum treated strain of Type I and found that it is agglutinated *in vivo* and *in vitro* by normal rabbit serum, while the control strain is not.

Variations in Virulence.

Simultaneous with the change in agglutinability, the immune serum treated cultures showed a pronounced loss of virulence for white mice and rabbits. It often required a million times more of an immune serum treated culture to kill than of the control normal serum treated culture. Virulence tests were always made with cultures at least twice removed from the serum treated culture in which 1 cc. contained only $\frac{1}{5,000}$ part of immune serum. As 0.1 cc. of this serum is required to protect against 0.1 cc. of a normal strain, this loss of virulence cannot be attributed to the protective value of the serum present. In rabbits it was found

that the washed bacteria from 15 cc. of a bouillon culture of treated organisms would not kill, while 0.000005 cc. of culture of untreated organisms killed in 24 hours. In white mice it often required the bacteria from 5 cc. of a culture of treated organisms to kill, while 0.000001 cc. of culture of untreated organisms killed in 24 hours.

Table III shows the comparative virulence of the immune and normal serum treated cultures of Pneumococcus Type I and Pneumococcus Type II, the agglutination tests of which are given in Tables I and II. The virulence of Subculture I 114 B, treated 12 times with normal serum was such that 0.000001 cc. of a 20 hour bouillon culture killed in 56 hours, while the fatal dose of Subculture A, treated 12 times with Immune Serum I, was 0.5 cc. of a 20 hour bouillon culture. After 20 treatments with immune serum, 1 cc. of a broth culture of Subculture A did not kill, and after 46 treatments the

TABLE III.

Virulence of Serum Treated Strains of Pneumococci.

The tests were made by intraperitoneal injection into white mice. All serum cultures were transferred at least twice before injection into such amounts of bouillon that the dilution of serum in the culture used was at least 1:5,000. Autopsies were performed on all animals and smears were made from the peritoneal exudate. Pneumococci were found in all cases.

Test I.

Virulence, Pneumococcus Type I.

Amount of culture injected.	12 serum treatments.		20 serum treatments.		46 serum treatments.	
	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.
cc.						
5.0	—	—	—	—	D. 18 hrs.	—
1.0	—	—	S.*	—	S.	—
0.5	D. 24 hrs.	—	S.	—	S.	—
0.1	S.	—	S.	—	—	—
0.01	S.	—	S.	—	—	—
0.001	S.	—	S.	—	—	—
0.0001	S.	D. 18 hrs.	S.	D. 26 hrs.	—	D. 36 hrs.
0.00001	D. 24 hrs.†	" 56 "	—	" 25 "	—	" 36 "
0.000001	—	" 56 "	—	" 25 "	—	" 36 "

TABLE III—*Concluded.*

Test II.

Virulence Tests of Serum Treated Pneumococcus Type II Made with Cultures Which Had Received 27 and 61 Transfers in Plain Bouillon Subsequent to 55 Serum Treatments.

Virulence, Pneumococcus Type II.

Amount of culture injected.	27 transfers in plain bouillon.		61 transfers in plain bouillon.	
	Treated in Immune Serum II.	Treated in normal serum.	Treated in Immune Serum II.	Treated in normal serum.
cc.				
2.0	D. 18 hrs.	—	—	—
1.5	—	—	S.	—
1.0	S.	—	S.	—
0.5	D. 36 hrs.	D. 18 hrs.	S.	—
0.1	S.	" 18 "	S.	—
0.01	S.	" 36 "	—	—
0.001	S.	" 24 "	—	—
0.0001	—	" 24 "	—	D. 23 hrs.
0.00001	—	" 20 "	—	" 48 "
0.000001	—	—	—	" 48 "

* In the tables D. stands for died; S., for survived. The figures represent the number of hours after injection before the death of the animal.

† No pneumococci were found in the heart's blood of this animal.

animal receiving the bacteria from 1 cc. of a broth culture of Subculture A survived. Subculture B, however, which was treated an equal number of times with normal serum, maintained a high virulence, 0.000001 cc. of a broth culture killing regularly.

In Test II, Table III, is shown the comparative virulence of an immune and normal serum treated culture of Pneumococcus Type II 34. Each culture had received 55 serum treatments and had been passed subsequently through a large series of broth transfers. After 27 transfers in plain broth the fatal dose of the immune serum treated culture was 0.5 cc., or more, while 0.00001 cc. of the normal serum treated strain killed a mouse in 20 hours. After 61 transfers in plain bouillon 1.5 cc. of the immune serum treated culture failed to cause death, although 0.000001 cc. of the normal serum treated culture was fatal to a mouse in 48 hours.

It will be seen from these tables that the longer the pneumococcus is grown in immune serum the less virulent it becomes, while the same strain grown in normal serum retains its original virulence even after long periods of growth in plain bouillon. The immune serum treated organisms retain the avirulent characteristics after as many as 75 transfers in plain bouillon subsequent to serum treatment. This fact demonstrates conclusively that the loss of virulence is not due to the protective action of the immune serum present.

A study of the capsule formation in the immune and normal serum treated cultures demonstrated the interesting fact that while the latter upon injection into mice formed capsules easily demonstrable by the Hiss method, the immune serum treated organisms showed no demonstrable capsules under similar conditions. It is possible that the loss of virulence noted may in some way be related to the apparent absence of capsule formation.

Variations in Phagocytability.

The phagocytability of the organisms grown in immune serum and of those grown in normal serum was tested with guinea pig leukocytes in the presence of normal and immune horse serum by the Neufeld method. The organisms grown in normal serum were phagocyted only in the presence of immune serum. Those grown in immune serum after at least two subsequent broth passages were phagocyted in the presence of both immune and normal horse serum. The organisms grown in immune serum were also phagocyted *in vivo* in the normal rabbit, a reaction which does not take place with the normal virulent pneumococcus. Growth in immune serum has, therefore, made susceptible to the phagocytic action of guinea pig leukocytes a culture of pneumococcus previously resistant to such action. This phenomenon may also be related to the loss of virulence which occurs as the result of growth in immune serum, since it is known that non-virulent pneumococci are more readily phagocyted than highly virulent strains.

Variations in Antigenic Properties.

In order to test the possibility of variation in the antigenic properties of immune serum treated strains, two antigens were prepared in the following manner. Emulsions of washed bacteria, killed by heating at 56°C. for 45 minutes, were prepared from equal amounts of two bouillon cultures of Pneumococcus Type II 34, one of which had been treated for 60 successive transfers with Serum II, the other for the same number of transfers with normal horse serum. Immune sera were prepared from these antigens by intravenous injection of rabbits. The immunization of each animal was carried out in a corresponding manner, and the serum obtained for the tests at comparable intervals of time. The immune rabbit sera thus prepared were tested for their agglutination reaction with normal and immune serum treated pneumococci of both Types I and II.

Examination of Table IV shows that the immune response as measured by agglutinins is slower in the rabbits immunized with immune serum treated pneumococci than in those immunized with the normal serum treated organisms. It is further evident that the serum of rabbits immunized to immune serum treated Pneumococcus Type II contains agglutinins for the immune serum treated organisms of both Types I and II, but does not possess antibodies for the strains treated

TABLE IV.

Agglutination Reaction of Sera from Rabbits Immunized to Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Agglutination Reaction of Sera Obtained 4 Weeks after the Beginning of Immunization.

Culture used for agglutination.	Serum from rabbits immunized with immune serum treated Pneumococcus Type II.		Serum from rabbits immunized with normal serum treated Pneumococcus Type II.	
	Rabbit 29C	Rabbit 30C	Rabbit 31C	Rabbit 32C
Normal Pneumococcus Type II...........	−	−	++	++
Immune serum treated Pneumococcus Type II..............................	−	−	±	±

TABLE IV—*Concluded.*

Agglutination Reaction of Sera Obtained 6 Weeks after the Beginning of Immunization, Including Cross Agglutination Reactions with Normal and Immune Serum Treated Pneumococci Types I and II.

Dilutions of serum.	Serum from rabbit immunized with immune serum treated Pneumococcus Type II.				Serum from rabbit immunized with normal serum treated Pneumococcus Type II.			
	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.
Undiluted.	−	+	−	+	++	+	−	+
1:10	−	+	−	+	++	+	−	+
1:20	−	+	−	+	++	+	−	+
1:40	−	+			+	+		
1:50		+			+	+		
1:100		+			+	+		
1:200		+			−	+		
1:400		−			−	−		

with normal serum. On the other hand, the sera of rabbits immunized to a Type II pneumococcus which had been grown in normal serum for a similar number of transfers, agglutinated not only the homologous normal strain, but reacted, although less sharply, with immune serum treated pneumococci of both Types I and II.

Variations in Absorption Properties.

It has been demonstrated by Avery (17) that absorption of Antipneumococcus Serum I or II with the homologous pneumococcus removes from the serum all agglutinins for the homologous organism, while saturation of Serum I with a Type II organism does not remove the agglutinins present for Type I, and the same result is obtained when an attempt is made to exhaust the antibodies of Serum II with Pneumococcus Type I. Saturation of the Antipneumococcus Serum I or II with homologous immune serum treated organisms, removes all agglutinins for both homologous and heterologous immune serum treated pneumococci, but does not remove the agglutinins for the homologous normal serum treated organisms. It is also found that saturation of antipneumococcus serum with

the homologous type of normal serum treated organisms removes the agglutinins not only for these pneumococci, but for all immune serum treated strains.

Comparative Absorption of Antipneumococcus Serum with Immune Serum Treated and Normal Serum Treated Pneumococci.

Technique.—To 5 cc. of Antipneumococcus Serum II was added the washed bacterial residue from 25 cc. of a 20 hour bouillon culture of Pneumococcus Type II which had been treated for 65 transfers with Immune Serum II. The mixture was kept at 37°C. for 30 minutes and for 18 hours at ice temperature. A similar mixture was prepared by adding to the same amount of Serum II the bacterial residue from 25 cc. of a 20 hour bouillon culture of a strain of Pneumococcus Type II which had been treated for 65 transfers with normal horse serum, and this mixture was also kept at 37°C. for 30 minutes and for 18 hours at ice temperature. At the end of that time both mixtures were centrifugalized and the clear supernatant fluids again absorbed with fresh bacterial residues of the immune and normal serum treated cultures. Agglutination tests were then made with these absorbed sera.

TABLE V.

Agglutination of Antipneumococcus Serum II before and after Absorption with Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Strains used for agglutination.	Before absorption.	After absorption with normal serum treated Type II.	After absorption with immune serum treated Type II.
Pneumococcus Type II, normal serum treated	++	−	++
Pneumococcus Type II, immune serum treated	++	−	−
Pneumococcus Type I, normal serum treated	−	−	−
Pneumococcus Type I, immune serum treated	+	−	−

Table V gives the result of an absorption experiment with Antipneumococcus Serum II. Absorption was repeated until the normal serum treated organisms had absorbed from the serum all the agglutinins for this strain. This serum then had lost, also, the agglu-

tinins for the immune serum treated organisms which it formerly contained, while the serum which had been absorbed with equal amounts of the immune serum treated organism, although it had lost the agglutinins for the homologous immune serum treated pneumococcus, still retained an apparently undiminished quantity for the homologous normal serum treated organism.

Reversion to Type of the Altered Strains on Animal Passage.

Attempts were made to ascertain whether these variations in agglutinability, virulence, and capsule formation would persist after repeated animal passage. This study revealed the fact that all strains so far tested reverted to the original type by passage through the animal body, the number of passages required for this reversion depending upon the number of treatments in immune serum which the organism had received. Strains which had received from 6 to 12 treatments regained virulence and specific agglutinability upon 1 animal passage, while it required 3 or more successive passages to restore the virulence and agglutinability of strains which had received from 50 to 100 serum treatments.

TABLE VI.

Effect of Animal Passage upon Agglutination and Virulence of Serum Treated Pneumococci.

Test I.

Agglutination and Virulence Tests Made before and after Animal Passage of Culture I 112^9, Which Had Received 6 Serum Treatments and 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
	Virulence of organisms treated in Immune Serum I.		Virulence of organisms treated in normal serum.	
cc.				
1.0	D. 16 hrs.	D. 18 hrs.	D. 16 hrs.	—
0.2	" 40 "	" 18 "	" 16 "	—
0.1	" 40 "	" 18 "	" 16 "	D. 18 hrs.
0.01	S.	" 18 "	" 20 "	" 18 "
0.001	S.	" 40 "	" 24 "	" 20 "
0.0001	S.	" 22 "	" 40 "	" 20 "

TABLE VI—*Continued.*

Agglutination of Culture I 112[9], before and after Animal Passage.

Serum used for agglutination.	Culture treated in Immune Serum I.		Culture treated in normal serum.	
	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
Immune Serum I	+	++	++	++
" " II	±	–	–	–
Normal horse serum	++	–	–	–

Test II.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture I 114[62], Which Had Received 59 Serum Treatments and Subsequently 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.
Virulence of organisms treated in Immune Serum I.			
Bacteria from 10 cc. culture	D. 16 hrs.	–	
" " 5 " "	" 16 "	–	
2.0 cc. culture	" 16 "	D. 16 hrs.	D. 36 hrs.
1.5 " "	S.	S.	–
1.0 " "	S.	S.	S.
0.5 " "	S.	–	–
0.1 " "	–	S.	–
0.001 " "	–	–	S.
Agglutination in Serum I	+	+	++
" " " II	±	±	–
" " normal serum	±	+	–
Virulence of organisms treated in normal serum.			
0.01 cc. culture	D. 36 hrs.	–	
0.001 " "	" 20 "	–	
0.00001 " "	" 25 "	D. 22 hrs.	
0.000001 " "	" 36 "	" 21 "	
Agglutination in Serum I	++	++	
" " " II	–	–	
" " normal serum	–	–	

TABLE VI—*Concluded.*

Test III.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture II 34[124], Which Had Received 109 Serum Treatments and Subsequently 4 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.	Result after third animal passage.
Organisms treated in Immune Serum II.				
Bacteria from 10 cc. culture....	D. 36 hrs.	—	—	—
" " 5 " "	S.	—	—	—
2.0 cc. culture	—	D. 18 hrs.	—	—
1.0 " "	S.	" 18 "	—	—
0.5 " "	—	" 18 "	—	—
0.1 " "	—	" 18 "	D. 16 hrs.	—
0.001 " "	—	" 18 "	—	D. 36 hrs.
0.0001 " "	—	" 36 "	D. 16 hrs.	" 36 "
0.00001 " "	—	—	" 20 "	" 36 "
0.000001 " "	—	—	" 24 "	" 36 "
Agglutination in Serum I.......	+	+	+	—
" " " II......	++	++	++	++
" " normal serum.	+	—	—	—
Organisms treated in normal serum.				
0.01 cc. culture...........	D. 16 hrs.	—		
0.001 " "	" 16 "	—		
0.00001 " "	" 20 "	D. 24 hrs.		
0.000001 " "	" 22 "	" 36 "		
Agglutination in Serum I......	—	—		
" " " II.....	++	++		
" " normal serum.	—	—		

Table VI shows the result of animal passage of three different strains of immune and normal serum treated pneumococci. Test I shows the result of 1 animal passage of a Pneumococcus Type I which had received 6 serum treatments, original virulence and typical agglutinability being regained upon 1 animal passage. Before passage it required 0.1 cc. of culture of the immune serum treated organism to kill mice in 40 hours, and the organisms were agglutinated with Serum II and normal serum; after passage 0.0001 cc. of culture killed

in 22 hours and the bacteria were agglutinated only in Serum I. Test II shows the result of 3 animal passages of a Pneumococcus Type I which had received 59 serum treatments. In this case it required 3 animal passages to cause the strain to revert to original agglutinability, and the virulence had not been materially raised by this number of passages. Test III shows the result of 4 animal passages of a Pneumococcus Type II which had received 109 serum treatments. With this strain 4 passages were required to restore the original agglutinability, although the virulence was raised by 1 passage. In all cases control tests were made with a normal serum treated organism, which had neither lost in virulence nor in specific agglutinability. A great number of similar tests have demonstrated that these immune serum treated strains are readily caused to revert to the original type by animal passage.

DISCUSSION.

The effect of the treatment of pneumococci with immune serum may be attributed to a suppression of certain receptors, similar to the phenomenon observed by Cole with inagglutinable typhoid strains, since the serum treated pneumococci become less specifically agglutinable, can no longer absorb the agglutinins for the normal strains, and, when injected into the animal body, do not produce agglutinating sera for the normal strains.

The effect produced on the virulence of the pneumococcus by immune serum treatment is not identical with that produced in other bacteria by treatment with immune sera. The pneumococcus, instead of becoming more virulent, (serum-fast or immune) with immune serum treatment, becomes much less virulent. This loss of virulence is one of the most noticeable effects of the treatment with the immune serum and, as mentioned before, cannot be explained by the protective action of the serum present, since it persists after 60 or 70 transfers in plain bouillon subsequent to serum treatment, when the amount of serum present in the culture is too small to compute, and after the organisms have passed through so many generations that there could surely be no immune substances from the serum still adherent to them. It would seem that some biologic change must have

taken place in the organism, transforming it from the virulent to the avirulent type. This inference is confirmed by the fact that these strains rendered avirulent have lost capsules and are phagocyted in normal serum.

At present we have no explanation to offer in regard to this difference between the virulence of the typhoid bacillus and the pneumococcus when grown in their immune sera. Since antityphoid serum is bactericidal and reacts best in the presence of complement, and since antipneumococcus serum possesses no demonstrable bactericidal properties and its agglutinating and protective action is not increased by the addition of complement, it is possible that the different results of serum treatment upon these bacteria with such widely separated biologic characteristics may, in some way, be attributable to the differences in the properties of these two sera.

It is interesting to note that the variations in pneumococci produced by treatment with immune serum do not persist after animal passage. Very few passages through the animal body cause the changed organism to revert to the original type with characteristic agglutinability, virulence, etc.

CONCLUSIONS.

1. The growth of virulent pneumococci in homologous immune serum produces (*a*) variations in agglutinability, (*b*) decrease in virulence, (*c*) inhibition of capsule formation, (*d*) increase in phagocytability with normal serum, and (*e*) change in absorption power and antigenic properties.

2. Reversion to the original type in these changed forms takes place upon animal passage.

BIBLIOGRAPHY.

1. Metchnikoff, E., *Ann. Inst. Pasteur,* 1887, i, 42.
2. Charrin and Roger, *Compt. rend. Soc. biol.,* 1892, iv, 620, 924.
3. Roger, G.-H., *Compt. rend. Soc. biol.,* 1890, ii, 573.
4. Metchnikoff, *Ann Inst. Pasteur,* 1892, vi, 297.
5. Sanarelli, *Ann. Inst. Pasteur,* 1893, vii, 225.
6. Issaeff, B., *Ann. Inst. Pasteur,* 1893, vii, 260.
7. von Ransom and Kitashima, *Deutsch. med. Woch.,* 1898, xxiv, 295.

8. Müller, P. T., *Münch. med. Woch.*, 1903, l, 56.

9. Eisenberg, P., *Centr. Bakteriol., 1st Abt., Orig.*, 1903, xxxiv, 739; 1908, xlv, 134, 638.

10. Walker, E.W. A., *J. Path. and Bacteriol.*, 1903, viii, 34.

11. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 270; 1903–04, x, 89.

12. Feiler, M., *Z. Immunitätsforsch., Orig.*, 1916, xxiv, 411.

13. Joos, A., *Centr. Bakteriol., 1st Abt., Orig.*, 1903, xxxiii, 762.

14. Cole, R., *Z. Hyg.*, 1904, xlvi, 367, 371.

15. Friel. *Pub. South African Inst. Med. Research*, Jan. 26, 1915.

16. Bull, C. G., personal communication.

17. Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

THE CONDITIONS AND CHARACTERS OF THE IMMUNITY PRODUCED IN THE GUINEA PIG BY INSTILLATION OF HORSE SERUM INTO THE NOSE.

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In a recent communication[1] we showed in detail that when a few drops of horse serum are instilled, on two or more occasions, into the nostrils of a guinea pig the animal may become affected in radically different ways. An intravenous injection of the antigen given 16 days after the last instillation may lead to more or less profound shock or speedy anaphylactic death. But in a certain proportion of cases the toxic injection produces no obvious reaction. It would appear that the animal had not absorbed the serum introduced into the nose were it not that a second toxic injection, given 24 days after the first, may also be withstood. Therefore, we must conclude that the guinea pig was made primarily refractory, not sensitive to the dose of horse serum introduced into the vein. It seemed to us important to determine the experimental conditions according to which a series of nasal instillations of serum would, on the one hand, render the guinea pig hypersusceptible, or, on the other, insusceptible to a toxic dose of the serum. Our work last year was based on the hypothesis that the biological results of the nasal treatment depended on the time intervals between successive instillations. But, although it became plain that the rhythm of dosages by the nose was not a matter of indifference, we were unable to explain by it the variable effects of the toxic injection.

A more favorable issue has attended the present series of experiments which was suggested through an occurrence which implied a definite relation between the amount of antigen and its qualitative

[1] Sewall, H., and Powell, C., *Arch. Int. Med.*, 1915, xvi, 605.

effects. Immediately, therefore, protocols were prepared for the investigation of the influence of the quantity of serum introduced into the nose upon the specific reactivity of the guinea pig.

The Qualitative Effects of Serum Introduced into the Nose Depend upon the Quantity Instilled.

Our experiments were performed upon young animals, averaging at the outset about 300 gm. in weight. The undiluted Cutter's horse serum was dropped into the nostrils of the animals in the manner previously described.[1] Each drop represented approximately 0.02 cc. The first intravenous or toxic injection, of 0.38 cc., was uniformly given 16 days after the last instillation; the second intravenous injection usually followed the first in 14 days.

Some excerpts from our general results are collected in Table I. The animals in each group were simultaneously carried through

TABLE I.

The Influence of the Quantity of Serum Instilled on the Reaction to Toxic Injections.

Group of guinea pig.	No. in each group.	Amount of serum used in each instillation.	Total no. of instillations.	Intervals between instillations.	No. surviving 1st intravenous injection of 0.38 cc. serum.	No. surviving 2nd intravenous injection of 0.38 cc. serum.
		cc.		*days*		
I	3	0.2	4*	1	0	
II	4	0.2	6†	2	0	
III	2	0.2	4	2	1‡	1
IV	6	0.1	6	2	4	2
V	2	0.04	4	2	2	2
VI	6	0.04	6	2	6	3
VII	6	0.04	12	1	5	3
VIII	6	0.02	12	1	6	4

* A fifth instillation of 0.2 cc. was given 14 days after the fourth.

† A seventh instillation was given 14 days after the sixth and the toxic injection delayed correspondingly.

‡ The amount of serum in the first intravenous injection was reduced to 0.25 cc.

identical procedures. It will be observed that four separate instillations of serum are sufficient to provoke a maximal biologic response. Of nine guinea pigs receiving instillations of 0.2 cc. in quantity (Groups I, II, and III), only one survived the first toxic injection and in this case the amount of serum given by the vein was reduced to 0.25 cc. From the reaction manifested by the animal it is probable that it also would have succumbed to the usual injection of 0.38 cc.

Of twenty guinea pigs receiving instillations of 0.04 cc. or less only one succumbed to the first intravenous injection of 0.38 cc. of horse serum. Twelve of these twenty animals withstood a second intravenous injection of 0.38 cc. of serum 14 days or more after the first. There can be no doubt, therefore, that three-fifths of the animals were strongly immunized by the preliminary instillations of serum. With regard to the seven guinea pigs which succumbed to the second toxic injection the question arises: Did they completely fail to absorb the serum instilled and thus become sensitized by the first toxic injection in the ordinary way, or was the protection conferred by the instillations insufficient in degree to balance the shock of the large second injection? The following considerations led us to adopt the latter explanation.

Many observations have impressed us with the conclusion that the fatality attending the second toxic injection is roughly proportionate to the degree of anaphylactic reaction manifested with the first.

In reviewing our notes we find that of 81 guinea pigs in which record was made of the degree of reaction manifested to intravenous injections following a preliminary course of nasal instillations, in eighteen cases little or no definite shock was caused by the first intravenous injection; of these only three animals succumbed to the second injection of 0.38 cc. of serum, a mortality of less than 17 per cent. Of thirty-nine animals surviving the first intravenous injection after greater or less shock no less than twelve succumbed to the second injection, a mortality of more than 30 per cent.

Another reason for believing that some degree of absorption attends the application of minute quantities of foreign serum to the mucous membrane of the nose consists in the fact that invariably a secretion of saliva follows within a few seconds the fall of a single drop of serum into the nostril; when the lower lip of the animal is

depressed saliva is seen to accumulate about the incisor teeth, provided it has not been swallowed as formed. This has been assumed by us to indicate absorption.

If the death of our animals with the second toxic injection is due, as we suppose, not to inertness of the nasal instillations of serum but to the insufficiency of their immunizing power, it should be easy to demonstrate a weak protective power in the instillations by reducing the amount of the second toxic injection. This we have done to some extent and have found that when the quantity of serum in the second toxic injection is reduced one-half the animals usually survive. There still remains, however, a certain proportion of animals in which the grade of immunity is still much too low to resist this amount of antigen. It is an impression gained from many experiences, but not especially investigated, that the mortality from the second intravenous injection is higher, other things remaining the same, when the second injection follows the first after an interval of 14 days rather than 24 days, the interval formerly employed by us.

The Amount of Serum Absorbed and Its Biologic Effect Depend upon the Method of Instillation.

In all our experiments the complex living mechanisms responded exactly, as depicted in Table I, to the crude method we employed to introduce the serum into the body. In our confirmatory experiments a series of irregularities fortunately developed, which, when investigated, led to a better understanding of the conditions of biologic response. Thus, in a group of six guinea pigs instilled six times on alternate days with 0.2 cc. of serum, it was expected that all would succumb to the toxic injection of 0.38 cc. given 16 days after the last instillation. On the contrary, three of the animals survived, one after very severe shock and two with little or none. The second intravenous injection was reduced to 0.19 cc., and given 30 days later. The animal which had been previously shocked died, the other two easily survived.

Again six guinea pigs were instilled as above, but the quantity of serum used at each instillation was reduced to 0.04 cc. It was expected that all would survive the first intravenous injection of 0.38

cc. of serum. On the contrary, four animals succumbed to it. It was then realized that the method of instilling the serum had been radically different in the two sets of experiments. In the first case the procedure of instillation had been hurried; in the second it was prolonged and attended by obvious vital reactions, such as abundant salivary secretion. It was necessary to determine whether our results could be definitely modified by varying the area of contact between the serum and mucous membrane.

When the head of the animal receiving the instillation is held with its long axis in a vertical plane the serum is probably confined to the respiratory canal and does not reach the turbinate mucous membrane. But if, while the guinea pig is held in the supine position, the head is well extended, the opportunity is given for the serum to gravitate through the complex turbinate convolutions, and this to an extent dependent on the time during which the posture is maintained.

Accordingly, two groups of four guinea pigs each were prepared to test this reasoning. All the animals received five instillations of 0.1 cc. of serum on alternate days. But in the first group the heads of the animals were held vertically and the serum was dropped quickly, from 15 to 30 seconds being consumed in administering the five drops of each dose. In the second group of animals the heads were held well extended and from 3 to 5 minutes were occupied in each instillation. The first toxic injection of 0.38 cc. of serum was given after the usual interval. All four guinea pigs of the first group easily survived; three of the second group died and the remaining one was strongly shocked. We therefore conclude that the biologic effect of a given dose of serum depends chiefly upon the extent of its contact with the mucous membrane of the turbinate apparatus.

It is probable that several other factors, including quality and temperature of the horse serum, take part in determining the coefficient of absorption.

As will be shown in the following section, great individual differences may distinguish animals in their reaction to the same treatment. The susceptibility to anaphylaxis of different families of guinea pigs is noteworthy. Thus, twelve belonging to a wholly different stock from that from which most of our animals were obtained

during the present year proved peculiarly sensitive to serum treatment. Three of them instilled four times with 0.2 cc. of serum on alternate days succumbed to the usual toxic injection, as was to have been expected. The other nine animals received like treatment but with only 0.04 cc. of serum in each instillation. The first intravenous injection of 0.38 cc. of serum killed one and shocked the remainder to a considerably greater degree than usual under these conditions.

Gradations of Sensibility Induced by Serum Instillations.

The reaction to an antigen exhibited by an immunized animal is expressed by a ratio one factor of which is the vital resistance of the host and the other the amount and virulence of the antigen.

In terms of physiology, the plane of immunity is determined by the threshold of irritability to the antigen. This has recently been well brought out by Webb[2] who has successfully inoculated guinea pigs with many thousands of tubercle bacilli of which the minimal lethal dose was 125; but he always failed to establish immunity against recent cultures of which the m.l.d. was only ten bacilli. When a guinea pig is submitted to a series of sensitizing instillations of horse serum it sometimes happens, in possibly 5 per cent of the cases, that with the terminal treatment 8 or 10 days following the first, the animal develops a pronounced attack of asthma with loud, moist bronchial râles.

This is a sign of intense sensitization; we have found the hypersensitiveness to persist at least 60 days and such animals invariably succumb to the first toxic injection as used by us. After three or four sensitizing instillations, another of the same kind repeated after the lapse of 2 weeks produces a greater or less respiratory disturbance in the majority of cases. Such animals succumb to the toxic injection, as do also some which have shown no asthmatic symptoms. Several of our animals which after courses of nasal instillation had been strongly immunized by intravenous injections of serum, when given a nasal instillation of serum after the lapse of some months, showed no reaction whatever. Formerly we were of the opinion that the expression of local sensitization as manifested by asthma required a defi-

[2] Webb, G. B., *J. Lab. and Clin. Med.*, 1916, i, 414.

nite incubation period of 8 or 10 days between the first nasal instillation and that which could produce asthmatic symptoms. Recently, however, we have found that in guinea pigs born of treated mothers and highly sensitized by the subcutaneous injection of 0.1 cc. of horse serum more than 3 months before, well marked asthma could be aroused by the first instillation of serum into the nose. The subject achieves peculiar practical importance in view of the analogous clinical asthma in human beings. Our demonstration of the variability of symptoms of local sensitization of the respiratory apparatus on a constant background of general sensitization makes it questionable as to how reliable tests performed on other peripheral mechanisms, such as the skin, may be for indicating the immunological state of the body as a whole.

The Earlier Instillations of a Series Determine the Biologic Effect of the Whole.

Such results as those depicted in Table I have led us to differentiate our dosages of serum into those which are protective and those which are sensitizing. Previous observations had led to the impression that the two biologic states could be developed one from the other by appropriate intranasal treatment. The following experiments indicate that such is not the case.

To four young guinea pigs were given by the nose six instillations of 0.04 cc. of serum on alternate days. This had been found to be a protective or immunizing treatment. In two of the animals the instillations were continued for four doses but the amount of the serum instilled was increased to 0.2 cc. The remaining two guinea pigs were allowed to rest 16 days and then were likewise given four nasal instillations of 0.2 cc. of serum. Both groups were given intravenous injections of serum 16 days after the last instillations and all the animals survived, with slight symptoms in some cases. A second intravenous injection of 0.38 cc. was likewise survived by all the animals except one of the first group.

In our previous work[1] we had found that as few as two instillations of serum were capable of rendering animals either fatally sensitive to a toxic injection or of inducing an immunity through which

they were able to resist a series of intravenous doses. In Table I several instances are given in which the biologic attitude is determined by a series of four instillations. This seems to us to be a matter of great practical importance and it will be expanded in the final discussion.

Inertness of Serum Administered by the Mouth.

An inconclusive but suggestive experiment was performed upon two guinea pigs by administering four instillations of serum orally. The serum, to the amount of 0.2 cc., was dropped under the tongue on alternate days. The animals showed no response whatever to the first toxic injection and died with the second. The conclusion is that the serum had not been absorbed from the mouth or had been so greatly diluted with saliva as to be ineffective, and that the animals became sensitized by the first injection.

The Biologic Effects of Serum Instilled into the Nose Are of Temporary Duration.

It is generally admitted that guinea pigs sensitized by subcutaneous or other parenteral avenues of injection retain their sensitiveness throughout life. In a former course of experiments we demonstrated fatal anaphylaxis in two guinea pigs that had received the last of a series of nasal instillations of serum 40 days before. But recent observations have indicated to us that immunizing phenomena established through the mucous membrane of the nose are of temporary duration. Two groups were treated, one with a series of six protective instillations of 0.04 cc. and the other with sensitizing doses of 0.2 cc. of serum. 51 days later intravenous injections of 0.38 cc. of serum were given to one animal of the former and two of the latter group. Slight disturbance was manifested by the last two animals, and all three animals died with the second toxic injection administered 15 days later.

Two guinea pigs were given a series of six protective instillations of 0.04 cc. of serum on alternate days. 93 days later each animal received an intravenous injection of 0.38 cc. of serum without response. 14 days later one was given a second intravenous injection

of 0.38 cc. and died. The other animal was given only 0.25 cc. and lived after a moderate reaction.

Two guinea pigs received six sensitizing instillations of 0.2 cc. on alternate days. The first intravenous injection of 0.38 cc. of serum given 51 days later was borne with slight response; a similar injection repeated in 15 days killed both animals.

Three guinea pigs were prepared by six instillations of 0.2 cc. of horse serum. One of these developed an attack of asthma with the last instillation. 60 days later a toxic injection of 0.38 cc. of serum killed the asthmatic guinea pig but produced slight response in the other two. 15 days later one of the remaining animals was given by the vein 0.19 cc. and succumbed after some resistance, the other was given 0.12 cc. of serum and survived with slight response.

Our conclusion is that the immunizing phenomena set up by unsupported nasal instillations of serum gradually disappear within about 3 months, the hypersensitiveness from large instillations first changing to a measure of resistance.

As will be seen later, when relatively large injections of serum are given by the vein to animals within the period of protection afforded by appropriate nasal instillations, the immunity produced is apparently permanent and intensified with time.

In Guinea Pigs Immunized after Preliminary Nasal Instillations the Immunity Is Strengthened with Lapse of Time.

In the paper referred to above we quoted the conclusions of Gay and Southard,[3] to the effect that artificial immunization produced by the injection of serum is but a condition of temporary refractoriness, full sensitiveness returning to the animals if they are kept long enough. How long this period must be is not stated, but our results with guinea pigs immunized through the nose and later treated with a series of intravenous injections of serum led to radically different conclusions. We showed reason to believe that when guinea pigs so handled were brought to resist easily the intravenous injection of a certain amount of serum, say 0.38 cc., with the lapse of time, at least up to 101 days, there was such an increase of refractoriness that

[3] Gay, F. P., and Southard, E. E., *J. Med. Research*, 1908, xviii, 407.

more than 1.0 cc. of serum given intravenously could be equally well tolerated.

We have confirmed our previous findings in the present investigation and at the same time studied a small number of controls immunized through the peritoneal cavity.

Table II gives the histories of fifteen guinea pigs, the last three of which are borrowed from the records of former work. Each of these animals had been prepared by a course of nasal instillations of horse serum, followed at stated intervals by intravenous injections of the same. In each column is recorded the number of days elapsing between the successive injections, and the whole time intervening between the last nasal instillation and the last intravenous injection is represented by the sum of these intervals, *e.g.*, in the case of Guinea Pig 4, this is 102 days. The amount of serum used at each injection is also noted, and the signs L_0 to $L++++$ indicate varying degrees of anaphylactic reaction, while D shows that death followed the dose given. Deferring consideration of Guinea Pigs 10, 11, and 12, we see that after a certain degree of resistance had been established, the tolerance of the animals against serum injected into the vein apparently progressively increased with the lapse of time without treatment. In Animals 1 to 3, inclusive, the nasal instillations of 0.2 cc. of serum plainly caused sensitization so that repeated small intravenous injections were necessary to establish a fair degree of tolerance. This is especially noticeable in Guinea Pig 3, which was one of a group of four animals similarly prepared, the others having succumbed early to small injections. Even so it is seen that an extraordinary increase of resistance develops with time after the fourth intravenous injection. It is obvious that there is some metabolic strain towards an equilibrium of increased resistance against disturbance by the antigen. A comparison of these cases with those of Table III, in which the preparation was by relatively large intraperitoneal injections of serum, shows that in the latter animals what we call the metabolic strain is towards an equilibrium of hypersensitiveness.

Guinea Pigs 4 to 15 of Table II were prepared by small nasal instillations such as we have found to confer primary protection. It is seen that the rule is that such a course of instillations reinforced by two intravenous injections is sufficient to establish a ten-

TABLE II.

The Influence of Time on the Development of Immunity in Guinea Pigs Prepared by Nasal Instillation and Tested by the Intravenous Injection of Horse Serum.

No. of guinea pig.	Preliminary treatment.	First intravenous injection. 1. Amount of serum. 2. No. of days since last instillation. 3. Result.	1. Amount of serum. 2. No. of days since last injection. 3. Result. Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.
1	Six instillations of 0.2 cc. serum on alternate days.	0.06 cc. 13 L++	0.06 cc. 23 L_0	0.19 cc. 16 L+	0.19 cc. 19 L	0.75 cc. 92 L++	0.75 cc. 114 L+
2	Same as No. 1.	0.06 cc. 13 L++	0.13 cc. 23 L+	0.25 cc. 16 L++	0.25 cc. 19 L+	0.75 cc. 92 L++	
3	Five instillations of 0.2 cc. at intervals of 1 to 4 days. (Very sensitive.)	0.02 cc. 15 L++	0.05 cc. 21 L++	0.13 cc. 16 L+++	0.13 cc. 19 L+	0.5 cc. 96 L++	0.75 cc. 125 L+
4	Six instillations of 0.1 cc. on alternate days.	0.38 cc. 16 L	0.38 cc. 16 L++++	0.75 cc. 70 L			
5	Six instillations of 0.04 cc. on alternate days.	0.38 cc. 16 L_0	0.38 cc. 16 L+	0.88 cc. 69 L+			
6	Same as No. 5.	0.38 cc. 16 L+	0.38 cc. 16 L++	0.81 cc. 69 D			
7	Same as No. 5.	0.36 cc. 16 L_0	0.38 cc. 16 L++	0.75 cc. 67 L++			
8	Six instillations of 0.04 cc. followed by four of 0.2 cc. on alternate days.	0.31 cc. 16 L+	0.38 cc. 14 D				

TABLE II.—*Concluded.*

No. of guinea pig.	Preliminary treatment.	First intravenous injection. 1. Amount of serum. 2. No. of days since last instillation. 3. Result.	1. Amount of serum. 2. No. of days since last injection. 3. Result.				
			Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.
9	Same as No. 8.	0.38 cc. 16 L	0.5 cc. 68 L+	0.75 cc. 122 L+++			
10	Twelve instillations of 0.02 cc. daily.	0.31 cc. 17 L	0.38 cc. 16 L++	0.75 cc. 64 D			
11	Same as No. 10.	0.38 cc. 17 L	0.38 cc. 16 L++	0.75 cc. 64 D			
12	Same as No. 10.	0.31 cc. 17 L_0	0.38 cc. 16 L++++	0.75 cc. 111 D			
13	Six instillations ± 0.15 cc. at intervals of 14 days.	0.38 cc. 16 L_0	0.38 cc. 24 L++	0.38 cc. 75 L_0	1.1 cc. 36 L+++		
14	Same as No. 13.	0.38 cc. 16 L++	0.38 cc. 24 L+++	0.38 cc. 75 L_0	1.13 cc. 101 L		
15	Same as No. 13.	0.38 cc. 16 L_0	0.38 cc. 24 L	0.38 cc. 75 L_0	1.13 cc. 101 L		

dency, elaborated with time, towards a greatly strengthened degree of immunity. Particular attention is called to a comparison of the histories of Guinea Pigs 8 and 9. Both had received essentially the same preparation but Guinea Pig 8 succumbed to the second intravenous injection of 0.38 cc. of serum 14 days after the first. Guinea Pig 9 was kept until 68 days after the first injection and then easily withstood 0.5 cc. of serum. Still more to the point is comparison of the records of Guinea Pigs 13 to 15. Each withstood the third in-

travenous injection of 0.38 cc. of serum without obvious reaction. 36 days later Guinea Pig 13 was given an intravenous injection increased to 1.1 cc. and nearly died. The remaining two animals did not receive their fourth intravenous injection until 101 days after the third; each then withstood with hardly perceptible shock the large amount of 1.13 cc. of serum by the vein. It will be noted that the reactions of Guinea Pigs, 10, 11, and 12 are wholly different from those described above. These animals received a preparatory nasal treatment of twelve instillations repeated at intervals of 24 hours instead of on alternate days. Several diverse experiences have led us to conclude that qualitative differences exist between the immunological response elicited in animals according as they receive successive instillations of serum within one or two or more days.

TABLE III.

The Influence of Time on the Development of Immunity in Guinea Pigs Prepared by Intraperitoneal Injection and Tested by Intravenous Injection of Horse Serum.

No. of guinea pig.	Preparation.	First intravenous injection. 1. Amount of serum. 2. No. of days since last intraperitoneal injection. 3. Result.	1. Amount of serum. 2. No. of days since last intravenous injection. 3. Result.					
			Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.	Seventh intravenous injection.
1	All animals were given six intraperitoneal injections of serum within a period of 10 days.	0.19 cc. 9 L++	0.2 cc. 22 L++++	0.31 cc. 16 L++	0.31 cc. 19 L+	0.31 cc. 78 L+	0.63 cc. 45 L++	0.63 cc. 116 L+++
2		0.31 cc. 9 L+	0.32 cc. 22 L+	0.38 cc. 16 L	0.38 cc. 19 L+	0.38 cc. 78 L	0.75 cc. 45 L+	0.75 cc. 116 L+++
3		0.2 cc. 32 L++	0.25 cc. 24 L++++	0.25 cc. 12 L	0.75 cc. 94 D			
4		0.2 cc. 32 L+	0.26 cc. 18 L+	0.25 cc. 18 L+	0.38 cc. 94 D			
5		0.23 cc. 32 L++	0.33 cc. 18 L	0.33 cc. 18 L++	0.38 cc. 95 D			

Table III represents the histories of five guinea pigs which received courses of intravenous injections of horse serum after a preparatory period in which six intraperitoneal injections of 0.5 cc. of serum were given within 10 days. Comparing the animals with Nos. 1, 2, and 3 of Table II we see that they were capable of tolerating a much larger initial intravenous injection than the latter, but in Table III the tolerance of the subjects to increasing doses of the antigen is shown to have increased more slowly than in Table II. But the fundamental difference is that the guinea pigs of Table III, after having been made relatively immune to a certain intravenous dosage of serum, when kept for 3 months or more and then reinjected, manifested an increase of susceptibility, whereas in Table II the contrary is the case under similar conditions.

It should be expected that Guinea Pigs 1 and 2 of Table III would have been thoroughly immunized by the long succession of intravenous injections. Nevertheless we find that when a seventh injection was given following a resting period of 116 days after the sixth the animals were much more profoundly shocked by the dose which had previously been fairly well tolerated. They would undoubtedly have succumbed to any such increase of dosage as was employed with impunity on the subjects of Table II.

General Hypersensitiveness Is Not Abolished by Intranasal Treatment.

Since it has been shown that immunity to toxic injections could be established by instillations into the nose of definite, small amounts of horse serum, that is, that intranasal treatment might be used to produce with certainty prophylaxis against anaphylaxis, it was important to discover whether the cure of a condition of serum hypersensitiveness might be effected in a similar manner. Several experiments were undertaken with this end in view in our work already reported. The results were uniformly negative; no animal which had been sensitized by subcutaneous injection of horse serum and was subsequently treated by nasal instillations of serum survived an intravenous injection of 0.25 cc. afterwards. Since learning the fundamental biologic importance of the amount of serum used in the instillations

it was thought well to repeat these experiments. Our results have thus far been uniformly negative, but in view of the practical importance of the subject the investigation is still being pursued.

DISCUSSION.

The results which have been described strengthen the hypothesis on which the work was founded; namely, that the mucous membrane of the nose is an avenue to the mechanism of immunity which offers peculiar advantages over parenteral routes. Nothing less should be expected if, as seems probable, nasal absorption is a normal stage in the development of natural immunity. We have shown that a guinea pig which has been treated by a series of four nasal instillations of 0.04 cc. of horse serum on alternate days may withstand 16 days later the relatively enormous toxic injection of 0.38 cc. with hardly perceptible reaction. It would appear that the instilled serum had not been absorbed by the nose were it not that a second toxic injection given 15 days after the first may likewise be easily tolerated.

Reasons have been given for believing that every application of as much as 0.02 cc. of horse serum to the mucous membrane of the nose of the guinea pig is attended with sufficient absorption to produce systemic effect. The biologic result of this absorption is qualitatively determined by the quantity of serum instilled and by the extent of mucous membrane with which it comes in contact.

The experiments seem to show that a few nasal instillations of serum quantitatively below a fairly definite minimum lead to a general elevation of the threshold of cellular irritability towards the antigen. Instillations of serum quantitatively in excess of a certain minimum induce, on the other hand, the opposite effect and lower the threshold of cellular irritability. In the first case, the advent into the body of an enormous increase in the amount of serum is tolerated with indifference; in the second case, a comparatively small toxic injection sets up vital reactions with a fatal outcome. Furthermore, it has been demonstrated that the direction in which the plane of metabolic irritability is shifted, and according to which the animal's sensitiveness to the antigen is decreased or increased, is determined by the first two to four of a series of separate instilla-

tions. Transferring these conceptions to the field of clinical experience, we find an explanation of many empirically determined truths.

No one will question the necessity of imposing absolute rest upon a member which has suffered an infected wound, an insistence on which has recently been made by Heidenhain.[4] The parting of the way to recovery or death is often marked by the signs of rest or use at the moment of injury. It is obvious that toxic absorption from the site of trauma must be quantitatively in somewhat inverse proportion to the quietude of the infected part.

If we apply to clinical conditions deductions drawn from our experiments with horse serum, the danger to the human organism from local infection lies not in the absorption of a lethal dosage of poison but in the fact that quantities of toxin in the circulation in excess of a certain minimum render the living cells hypersusceptible to the toxin and transform them to a state of disastrous reactivity (allergy). On the other hand, absorption of toxins in amounts below the critical minimum is not biologically indifferent, but progressively strengthens the resistance of the cellular protoplasm against the later onslaught of enormous doses of toxin. In short, we wish to indicate the necessity for rest in therapeusis.

Our experiments indicate that the elevation or depression of the plane of cellular irritability has been determined at the end of a day or two after inoculation with poisonous material; hence the peculiar value of the early application of the rest treatment. Heidenhain and others have pointed out the surpassing importance of rest at the beginning of a course of surgical infection. Abundant as is the clinical endorsement of similar treatment during the prodromal stages of all medical infections, we must express our doubt whether medical practitioners generally apprehend the value of that early quietude on the part of the patient, the therapeutic importance of which we have sought to establish on a rational basis. Medical infections, whether acute or chronic, undoubtedly involve the same principles as those set up by accident or intention. The necessity for rest and exercise as here set forth will find its effective censor in the practical clinician.

[4] Heidenhain, L., *Münch. med. Woch.*, 1915, lxii, 1482.

It may be suggested that in prophylactic vaccination, as against typhoid fever, a new importance is given to the choice of quantity of material employed in the initial dose. Finally, it must be clear that underlying these investigations is the desire to define more accurately the general principles of prophylaxis against infectious disease.

It is not improbable that choice of the nasal route as a channel of protective inoculation would be simply a return to ancient practice; it is said that the Chinese and Hindus long ago vaccinated against smallpox by blowing the powdered virus into the nose.[5] Today literature is beginning to show evidence of desultory use of the nasal mucous membrane as an avenue of inoculation against infection.[6]

We have been strengthened in the notion, suggested in our former paper, that the introduction of an antigen into the organism leads to the development of two antagonistic antibodies, one of which makes for anaphylaxis and the other against it. If this is true, it is evidently the characteristic property of nasal absorption to allow the easy propagation of one antibody in preference to the other. We think we have demonstrated the experimental conditions under which this can be done. It is a familiar fact that normal guinea pigs inoculated with the serum of hypersensitive animals become themselves passively anaphylactic. It may be proper to record here that, in a course of experiments still under way we have found, in cooperation with Mitchell, that the serum of our protected guinea pigs when inoculated into normal animals has, under certain conditions, been able to protect them from the effects of several intravenous injections of the antigen given at 14 day intervals.

SUMMARY.

1. Normal guinea pigs treated by four to six instillations of horse serum into the nose on alternate days become either hypersensitive or refractory to an intravenous injection of 0.38 cc. of serum given 16 days after the last instillation. If the amount of serum in each

[5] Klebs, A. C., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 69.

[6] See Paget, O., *Med. Rec.*, 1915, lxxxviii, 470. Herrman, C., *N. Y. State J. Med.*, 1915, xv, 233.

instillation is as much as 0.2 cc., anaphylactic death is caused by the toxic injection. If the amount of serum in each instillation is reduced to 0.04 cc. the first intravenous injection is without marked effect, and a second injection and subsequent injections of the same amount of antigen are well tolerated in about half the cases.

2. The effect produced by a given dose of serum, whether protective or anaphylactic, depends probably upon the extent of contact with the mucous membrane of the nose.

3. Guinea pigs which, after nasal treatment, have become tolerant to a definite maximum intravenous injection of the antigen appear to increase the degree of their tolerance, at least up to a resting period of more than 4 months. The same does not hold in animals immunized by the peritoneal route.

4. The first two or three instillations of a series probably determine the biologic character, whether of hypersensitiveness or hyposensitiveness, of reaction towards the serum.

5. It is probable that, contrary to the case in parenteral sensitization, hypersensitiveness and protection, respectively, set up by nasal instillations and not followed by parenteral injections, gradually disappear in about 50 to 100 days.

6. We have failed in attempts to eliminate hypersensitiveness, due to subcutaneous injection of serum, by nasal instillations which would protect the normal animal from the development of anaphylaxis.

7. It is suggested that the principles of prophylaxis evolved under these relatively simple conditions should be applied in the study of infectious disease.

CONCLUSION.

We deduce from our observations that the peculiar value of rest in the treatment of infection depends upon the fact that absorption of minimal amounts of toxic matter produces a positive protective reaction in the organism, while the absorption of larger amounts renders the cells hypersensitive. The biologic response to the intoxication is probably chiefly determined within the first 48 hours of absorption, and, therefore, rest at the beginning of an infective process has preponderant prophylactic value.

THE DIFFERENTIATION OF CELLS AS A CRITERION FOR CELL IDENTIFICATION, CONSIDERED IN RELATION TO THE SMALL CORTICAL CELLS OF THE THYMUS.

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PLATES 8 TO 10.

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Means of Cell Identification.

Until recently the morphological structure of a cell has furnished the chief criterion for its identification. Erythrocytes and leukocytes, nerve and muscle cells, and various other specifically differentiated cells exhibit in their structure sufficient characters for their identification.

The identification of groups of different cells connected locally and genetically is more difficult. In the hematopoietic organs of an adult organism, for example, there are located groups of different cells connected by uninterrupted stages of transition. The youngest and the least differentiated cells give rise to more highly differentiated cells, and at the same time increase their number by multiplication. The proliferation of the young stem cells and their simultaneous differentiation in various directions, according to environmental conditions, lead to considerable diversity between cells, coexisting in time and place; and the various stages of different cells may sometimes manifest merely slight and doubtful peculiarities in their structure.

In similar cases histo- and embryogenetic studies may be of assistance in the identification of various cells, and may establish a gradual differentiation of the cells, in accordance with different embryonic stages. They may also connect certain structures with definite external factors, and by this means greatly facilitate the identification of the various cells in the adult organism. There still remain many cases in which the identification of cells is doubtful.

The small round cells of the cortex of the thymus are an example of this.

It has recently been established that the form of the cell is not a characteristic of certain kinds of cells. The method of tissue culture, which gives an opportunity of observing directly the gradual changes, occurring in the cell *in vivo*, shows how unessential the form of the cell is for its inherent potencies, and how completely this form depends upon the physical conditions under which the cell lives.

Harrison[1] has shown that the nerve cells of frogs may be stimulated by solids, that they respond to solids by an orienting movement, that they adapt their form to that of the spider web, and, finally, that they become spherical within a large hanging drop. Uhlenhuth[2] has established the dependence of the form of the epithelial cell upon the density of the medium in which the cell is growing. In a soft medium the epithelial cell, according to Uhlenhuth, assumes a spindle form, which is generally considered a characteristic feature of connective tissue cells. On the other hand, a liquid medium makes the epithelial cell spherical in shape. Burrows[3] connects the shape of the cell with a distribution of metabolic products in the medium. Finally, Rous[4] has shown that the spindle-shaped cells of connective tissue growing *in vitro*, when liberated from the clot by means of trypsin, assume at once the spherical form. Thus the cell form in its latest conception is not a criterion for cell identification.

To what extent, therefore, has our conception regarding specifically differentiated cells to be altered? Is the cell form merely the reflection of the density of the medium or is a certain kind of cell form implied also by the chemical constitution of the cell? There is strong evidence for the assumption that the shape of the cell is the

[1] Harrison, R. G., The Reaction of Embryonic Cells to Solid Structure, *J. Exp. Zool.*, 1914, xvii, 521.

[2] Uhlenhuth, E., Cultivation of the Skin Epithelium of the Adult Frog, *Rana pipiens*, *J. Exp. Med.*, 1914, xx, 614; The Form of the Epithelial Cells in Cultures of Frog Skin, and Its Relation to the Consistency of the Medium, *ibid.*, 1915, xxii, 76.

[3] Burrows, M. T., The Tissue Culture as a Physiological Method, *Tr. Cong. Am. Phys. and Surg.*, 1913, 77.

[4] Rous, P., and Jones, F. S., A Method for Obtaining Suspensions of Living Cells from the Fixed Tissues, and for the Plating Out of Individual Cells, *J. Exp. Med.*, 1916, xxiii, 549.

result of external physical factors. On the other hand, the structural differentiation of the cyto- and karyoplasm seems to depend upon the chemical constitution of the cell and upon the environmental conditions to which the cell is submitted. The specific chemical constitution of the different cell groups is exhibited in their specific morphological structure and is manifested in the resting stage as well as in the mitotic stage. The differences in the constitution of the cells make them non-interchangeable even under equal conditions, at least under those found in the living organism.

The process of cell differentiation under definite conditions is always the same for certain kinds of cells. Therefore the manifestation of further differentiation potentialities by a cell, the nature of which is doubtful, may be used as a criterion for its identification.

The Small Thymus Cells.

The nature of the small thymus cells has long been a subject of controversy. These cells are considered by one group of investigators (Prenant,[5] Bell,[6] Stöhr,[7] and others) as derivatives of the epithelium and capable of again developing into true epithelial cells. In accordance with this conception, the form and the structure characteristic of a definite cell group, by means of which the cell unity of the small lymphocyte was easily identified (in the blood, in the loose connective tissue, in the bone marrow, in the lymph glands, and in the spleen), became an unessential and temporary attribute of cells, in reality entirely unrelated to the small lymphocytes.

Another group of investigators (Maximow,[8] Danchakoff,[9] and

[5] Prenant, A., Recherches sur le développement organique et histologique des dérivés branchiaux, *Compt. rend. Soc. biol.*, 1893, v, 546. Prenant and Saint Remy, Sur l'évolution des formations branchiales chez le lézard et l'orvet, *Compt. rend. Acad.*, 1902, cxxxv, 1.

[6] Bell, E. T., The Development of the Thymus, *Am. J. Anat.*, 1906, v, 29.

[7] Stöhr, P., Ueber die Natur der Thymus-Elemente, *Anat. Hefte*, 1906, xxxi, 407.

[8] Maximow, A., Untersuchungen über Blut und Bindegewebe. V. Über die embryonale Entwicklung der Thymus bie Selachiern, *Arch. mikr. Anat., Ite Abt.*, 1912, lxxx, 39.

[9] Danchakoff, V., Untersuchungen über die Entwicklung von Blut- und Bindegewebe bei Vögeln; das lockere Bindegewebe des Hühnchens im fetalen Leben, *Arch. mikr. Anat.*, 1908–09, lxxiii, 117; Ueber die Entwickelung der embryonalen Blutbildung bei Reptilien, *Verhandl. anat. Ges., Anat. Anz.*, 1910, xxxvii, Suppl., 70.

especially Hammar[10]) established a close connection between the development of the small thymus cells and an infiltration of the thymus by wandering cells, which proliferated and differentiated within the epithelial thymus anlage into small lymphocytes.

Most of the investigators of both groups based their conclusions on the histogenetic principle of the gradual differentiation of one class of cells from the other. Both groups of investigators supported their conclusions by the existence of transition stages between the differentiated small thymus cells and the cells from which they were derived. As a matter of fact, one group referred to transition stages between the small thymus cells and the invading mesenchymal elements, whereas the other mentioned no less definitely the presence of transition stages between the small thymus cells and the cells of the epithelial anlage, overlooking the infiltration of the anlage by wandering cells of mesenchymal origin. The structure of the small thymus cells, identical with that of the small lymphocytes, caused them to appear merely as a disguised form of a true epithelial cell.

The insufficiency of the histogenetic principle in the identification of the small thymus cells was obvious from the fact that in 1908 various workers in the Institute for Histology at Moscow simultaneously expressed opposite views. Gamburtseff[11] considered the small thymus cells to be epithelial elements; Danchakoff[12] established in the thymus of birds a differentiation of the small lymphocytes at the expense of lymphoid hemocytoblasts (large lymphocytes). Maximow's[13] histogenetic studies demonstrated the lymphatic nature of

[10] Hammar, J. A., Der gegenwärtige Stand der Morphologie und Physiologie der Thymusdrüse, *Congrès internat. méd., Budapest, Compt. rend.* Fünfzig Jahre Thymusforschung. Kritische Übersicht der normalen Morphologie, *Ergebn. Anat. u. Entwcklngsges.*, 1909, xix, 1.

[11] Gamburtseff, A., Die Histogenese der Thymus, Dissertation, Moskow, 1908. Cited by Hammar, J. A., *Ergebn. Anat. u. Entwcklngsges.*, 1909, xix, 274.

[12] Danchakoff, V., Untersuchungen über die Entwicklung von Blut- und Bindegewebe des Hühnchens im fetalen Leben, *Arch. mikr. Anat.*, 1908–09, lxxiii, 117.

[13] Maximow, A., Untersuchungen über Blut und Bindegewebe. IV. Über die Histogenese der Thymus bei Amphibien, *Arch. mikr. Anat., 1te Abt.*, 1912, lxxix, 560; V. Über die embryonale Entwicklung der Thymus bei Selachiern, *ibid., 1te Abt.*, 1912, lxxx, 39.

the thymus cells in mammals, amphibians, and selachians; the same was done by Danchakoff[14] in the case of reptiles.

The balance of evidence has of late appeared strongly to support the substitution theory, or the theory of the true lymphatic nature of the small thymus cells. However, this problem has not been conclusively solved.

The recourse to cell differentiation as to a criterion for cell identification is not altogether new. In 1909 Schaffer[15] stated that the transformation of the small thymus cells into plasma cells in the lobules of the thymus during involution of the organ may give proof of their true lymphatic nature.

The fact that the mother cells of the small thymus cells in reptiles also differentiate into numerous granulocytoblasts is accounted for by Danchakoff[16] (1910) by the lymphatic nature both of the small thymus cells and of their mother cells. Maximow also mentions the existence of a slight granulocytoblastic development in the thymus of mammals.

It is known that the small lymphocytes develop in part at the expense of the mesenchymal cells, but they appear chiefly as the result of an intense proliferation of the lymphoid hemocytoblasts. The mesenchymal cells, however, as well as the lymphoid hemocytoblasts, are similarly capable of differentiating under definite conditions into granulocytoblasts and granulocytes (granular leukocytes). This line of differentiation is absent in the epithelial cells.

The mother cells of the small thymus cells manifest distinctly in the normal embryos of reptiles (Danchakoff[16]) their power of differentiating into granulocytoblasts. This line of differentiation is, however, only slightly exhibited in birds. In the connective tissue septa but few granulocytoblasts are seen developing at the expense of lymphoid wandering cells. If by experimental intervention the line of differentiation of the mother cell of the small thymus cells may be inter-

[14] Danchakoff, V., Über die Entwicklung des Blutes in den blutbildenden Organen bei *Tropidonotus natrix*, *Arch. mikr. Anat.*, 1916, lxxxvii, 497.

[15] Schaffer, J., Über Thymus und Plasmazellen, *Zentr. Physiol.*, 1908–09, xxii, 422.

[16] Danchakoff, V., Ueber die Entwickelung der embryonalen Blutbildung bei Reptilien, *Verhandl. anat. Ges.*, *Anat. Anz.*, 1910, xxxvii, Suppl., 70.

changed, if it may be directed, for example, into the granulocytoblastic mode of differentiation (which is characteristic only of true lymphoid elements and never occurs in epithelial cells under conditions existing in the organism), it would follow that the small thymus cells are the offspring of true lymphatic elements and are not derivatives of the epithelial cells.

It is also known that the true small lymphocytes may under certain conditions differentiate into plasma cells, and, in the case of birds, into special wandering cells, characterized by the presence in their cytoplasm of numerous minute acidophylic granules. An intensive differentiation of the cortical cells into plasma cells in the thymus of the hen may be observed after the administration of small doses of x-ray to the animals. This differentiation appears undoubtedly as a proof of the lymphatic nature of the small thymus cells.

Finally, the present study has shown the existence of a physiological differentiation of the small thymus cells into granular lymphocytes, characteristic of birds, which indicates strongly the lymphatic nature of the small thymus cells.

Histogenetically the Small Thymus Cells Are the Offspring of Cells Morphologically Identical with the Lymphoid Hemocytoblasts.

The chief evidence for the lymphoid nature of the small thymus cells was recently shown by an invasion of the epithelial thymus anlage by wandering cells of mesenchymal origin. At the time of the first appearance of the small lymphocytes in the thymus of birds a striking picture of the differentiation processes is seen (Fig. 1). At this time the loose tissue of the thymus medulla merges gradually into the denser cortical layer. The parts of the cortical layer closely adjacent to the medulla appear also less dense than the parts surrounded at the periphery by the loose connective tissue. The cells constituting the cortical layer exhibit a marked difference as regards their structure. In the looser parts adjacent to the medulla numerous large ameboid cells are found, the structure of which is typical for the lymphoid hemocytoblasts (Fig. 1, L. Hbl.). Moreover, many of the cells seem to repeat on a miniature scale the structure mentioned (S. L. Hbl.). The nearer they approach the periphery of the organ,

the denser becomes the tissue and the smaller appear the lymphoid hemocytoblasts. And they tenaciously preserve the characteristic nucleus, which is relatively large, light, and spherical in shape, containing a well defined nucleolus. Though the cytoplasm of these cells diminishes, it still appears intensely basophilic. These cells may be called small lymphoid hemocytoblasts, and multiply profusely. They do not appear specific for the thymus, but arise in the organism where the lymphoid hemocytoblasts proliferate intensely in regions insufficiently supplied with nutritive material. Their appearance in the thymus must therefore be connected with the local agglomeration of the cells in relation to their intensive proliferation.

The new generations of the small lymphoid hemocytoblasts continue to multiply at the expense of the large lymphoid hemocytoblasts as well as by their own mitotic proliferation. The cortical layer of the thymus abounds at this time with mitoses. The decrease in size of the small lymphoid hemocytoblasts does not extend beyond a definite limit. Their further existence in the limited and poorly vascularized space, where the cells are crowded together, must lead to a change in metabolism. This change reveals itself by the differentiation of a new cell form, the small lymphocyte (S. Lmc.). The gradual differentiation of the small lymphocytes at the expense of the lymphoid hemocytoblasts is easily established in the embryonic thymus. A gradual accumulation of differentiated chromatin in the nuclei is discernible in the cells (Fig. 1, x). This accumulation is closely connected with a gradual exhaustion and a final disappearance of the nucleolus. The cells are ameboid and the narrow rim of cytoplasm becomes less basophilic. Fig. 2 shows a variety of transition stages (*a* to *f*) through which a large lymphoid hemocytoblast passes during its transformation into a small lymphocyte.

There is a striking difference in the mitoses (Fig. 3, *a*, *b*, *c*) of the two kinds of cells. It is difficult to establish the number of chromosomes, as their size is very minute. The size and the general aspect of the chromosomes, however, differ widely. The differences between the various kinds of mitosis, specific for certain types of cells, were repeatedly noticed. The peculiarities in the mitosis, appearing in connection with the cell differentiation, must not be overlooked, especially in view of the recent tendency to dissociate entirely the cell differen-

tiation from the factorial background of the cells, which is supposed to be the same in every cell of the body.

In his last book Morgan[17] states: "There is extensive evidence from cytology, experimental embryology, and regeneration, to show that all the different cells of the body receive the same hereditary factors" (which Morgan connects exclusively with the chromosomes). "We must suppose, then, that the Mendelian factors are not sorted out, each to its appropriate cell, so that factors for color go only to pigment cells, factors for wing-shape to cells of the wings, etc., but that differentiation is due to the cumulative effect of regional differences in the egg and embryo, reacting with a complex factorial background that is the same in every cell."

This statement finds support in histogenetic studies and in experimental embryology, but not to the extent outlined above. The hematopoietic studies corroborate the statement quoted above in demonstrating the existence of a lymphoid hemocytoblast, of which further differentiation into various kinds of blood cells is directed by the cumulative effect of regional differences. But there seem to exist in the different cells stages at which the chemical constitution and the metabolism of the cells are changed so essentially that the complex factorial background of the cell ceases to be the same for every cell. From this time on the cells are no longer interchangeable even under identical conditions; as, for example, in the blood stream. Under normal conditions the different kinds of blood cells within the vessels preserve unchanged their structural and functional peculiarities. Most of the cells present in the blood stream have lost their power of multiplication. Yet during leukemias the blood contains numerous less differentiated cells, endowed with mitotic faculty. They nevertheless do not interchange under identical conditions, to which they all are submitted in the blood current. In the hematopoietic organs, before the cells are fully differentiated and have lost their reproductive power, there appear differences in the mitotic process of their multiplication which are specific for certain kinds of cells.

With our limited knowledge and methods we cannot deny the exist-

[17] Morgan, T. H., Sturtevant, A. N., Muller, H. F., and Bridges, C. B., The Mechanism of Mendelian Heredity, New York, 1915, 43.

ence of functional differences between two morphologically identical cells. But from the time that certain groups of cells exhibit, both at the resting stage and during mitosis, well pronounced features characteristic only of these groups, the factorial background of the cell must have changed. If the complex of chromosomes, with which the Mendelian School exclusively associates the different factors, becomes visibly changed, a change in the factors of differentiation may be assumed. In view of a simultaneous restriction of differentiation potentialities in such cells a segregation of the differentiation factors may be assumed, if these factors are really as closely connected with the chromosomes as the Mendelian School supposes.

The establishment of a definite morphological structure for the small lymphocytes at rest as well as during mitosis seems to be an argument for an admission of a special differentiation of this cell unity. The small lymphocytes as such do not present the last stage in their evolution, developing under certain conditions into mast cells, into plasma cells, and into granular cells specific for birds. The analysis of the origin of the small cortical cells in the embryonic thymus shows that these cells must be regarded as derived from stem cells the structure of which is similar to that of the lymphoid hemocytoblasts.

The Mother Cell of the Small Thymus Cell Is a True Lymphoid Hemocytoblast. It Is Polyvalent and May Be Directed into the Line of Granulopoiesis.

The small thymus cell is looked upon by many histologists as a product of differentiation of the epithelial cells. The morphological structure of the small thymus cells, identical with that of the small lymphocytes of the blood stream and lymphatic organs, does not seem to be a sufficient argument to establish the true lymphatic nature of the thymus cells. Therefore it would be vain to suppose that the morphological structure of their mother cells, identical with that of the lymphatic hemocytoblasts, would be taken into account for the solution of the problem. The small thymus cells are considered by many investigators as being merely a disguised form of epithelial cell. The same assumption may be applied to their mother cells.

Stöhr[18] and Marcus[19] believe the small thymus cells to be capable of differentiating into true epithelial cells. Therefore the solution of the problem would depend upon the possibility of adding to the existing criteria new ones that are qualitatively different.

The study of hematopoiesis in reptiles has already given data relative to the potentiality of double differentiation which exists in the mother cells of the small thymus cells. These stem cells develop on the one hand into the small lymphocytes, and on the other hand into granulocytoblasts, and further into granulocytes (granular leukocytes). In birds and mammals this process does not seem to be of any importance. The polyvalence of the stem cells, which, in the thymus of reptiles, differentiate into cortical cells and granular leukocytes, is a strong argument for their true lymphatic nature and consequently for the lymphatic nature of both kinds of their daughter cells. If there is doubt of the identification of certain kinds of cells and of their mother cells, but if a possibility exists of tracing a characteristic line of differentiation of the polyvalent mother cells, the nature of these common mother cells is thereby solved and the identification of the other products of differentiation greatly facilitated.

On a recent occasion I had the opportunity[20] of demonstrating the polyvalence of the mesenchymal cells in embryos of 7 to 10 days, which revealed itself in a striking manner through the whole loose mesenchyme after spleen grafts on the allantois.[21] The specific line of granulocytoblastic differentiation, exhibited by the mesenchymal cells in different regions of the body, is also shown by the lymphoid hemocytoblasts of the thymus.

The anlage of the thymus appears in the form of separate foci of epithelial tissue, which at the beginning are independent. These agglomerations of epithelial cells develop in a full grown bird into large flat glands (0.5–1.5 cm. in length) which often join. They are located in the embryo, as well as in the adult animal, a little be-

[18] Stöhr, P., Ueber die Natur des Thymuselemente, *Anat. Hefte*, 1906, xxxi, 407.

[19] Marcus, H., Ueber die Thymus. Lebenslauf einer Thymuszelle, *Verhandl. anat. Ges.*, *Anat. Anz.*, 1907, xxx, Suppl., 237.

[20] Meeting of the Association of American Anatomists, New Haven, Conn., Dec. 28–30, 1915.

[21] Murphy, Jas. B., *J. Exp. Med.*, 1916, xxiv, 1.

hind the neurovascular bundle of the neck, and extend a little lower than the clavicle.

Macroscopically the grafting is followed by a visible enlargement of the thymus glands. This enlargement may be noticed as early as 2 days after grafting. On microscopic examination the thymus appears to be widely infiltrated by the large lymphoid hemocytoblasts, which proliferate intensely. The tissue of the thymus assumes a looser appearance, the limits between the cortex and the medulla disappearing almost completely. The infiltration by hemocytoblasts is equally strong in the cortex and medulla. Even at this early stage, after grafting, the lymphoid hemocytoblasts show conspicuously a tendency to differentiate in a granulocytoblastic direction and many of them develop in their cytoplasm numerous acidophylic granules. This line of differentiation of the hemocytoblasts is intensely developed in the thymus tissue 3 to 4 and 5 days after grafting (Fig. 4). One of the numerous protuberances covering the surface of the thymus is shown in the drawing. On the right of the figure is seen a part adjacent to the medulla. On the left are shown the peripheral layers of the thymus.

The cells forming the tissue of the thymus are different in structure. Besides a few reticulum cells, the cortical layer of the thymus contains large lymphoid hemocytoblasts (L. Hbl.), characteristic of this stage, and also small lymphocytes (S. Lmc.), which develop at the expense of the first mentioned cells. The differentiation of the small lymphocytes is, however, not intense, as in the normal thymus of the same stage (Fig. 1), and seems to be partly substituted by an intense development of granulocytoblasts and granulocytes (Fig. 4, Grbl., Grc.). This process of differentiation of the large hemocytoblasts undoubtedly corresponds to what is seen normally in the specific granulocytoblastic organs, chiefly in the annexes of the yolk sac (Danchakoff[22]), and in the bone marrow, and partly in early stages in the mesenchyme.

[22] Danchakoff, V., Untersuchungen über die Entwickelung des Blutes und Bindegewebes bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo und der Dottersack als blutbildendes Organ, *Anat. Hefte*, 1908, xxxvii, 471; Ueber die Blutbildung im Dottersack des Hühnchens, *Verhandl. anat. Ges., Anat. Anz.*, 1908, xxxii, Suppl., 72.

The development of the granulocytoblasts and their further differentiation into granular leukocytes is principally localized in the parts of the thymus where the tissue is looser. The tissue being less dense in the medulla and in the adjacent parts of the cortical layer, more numerous granulocytoblasts develop. The hemocytoblast here seems to find suitable conditions for growing into a large cell and for developing in its cytoplasm numerous acidophylic granules. A peculiar feature in the differentiation of granulocytoblasts after grafting is exhibited by pronounced differences in the size of the cells. It is possible that this is connected with the intensity of the process, spreading out over many cell generations at the same time. Most of the granulocytoblasts here are also large cells. The cells in Fig. 5, *a* to *f*, show the gradual transformation of a lymphoid hemocytoblast into a granulocytoblast and further into a granulocyte. The proliferation of the cells in the peripheral layers of the thymus is very intense, as is normally the case, and the hemocytoblasts gradually become smaller. The areas of rapid cell proliferation, accompanied by a diminution in cell size, are here, as normally, the first centers of differentiation of the small lymphocytes.

The transformation of the stem cells in the thymus into granulocytoblasts is an important argument for their true lymphatic nature and also for the lymphatic nature of the other products of differentiation of these stem cells; namely, of the small thymus cells. The same stem cells which under conditions of normal development produce a generation of small thymus cells now become the source of a most intense granulopoiesis. It is known that in normal development granulocytes and granulocytoblasts differentiate at the expense of a polyvalent lymphoid hemocytoblast, which under definite conditions becomes also the source of development of small lymphocytes.

The possibility of experimental deviation of the normal development of the mother cells in the thymus proves: (*a*) that the mother cells of the small thymus cells, being capable of differentiation also into granulocytoblasts, are true lymphoid hemocytoblasts; and (*b*) that the small thymus cells, being the offspring of true lymphoid hemocytoblasts, and exhibiting the structure of small lymphocytes, are true small lymphocytes.

That the mesenchymal cells and the lymphoid hemocytoblasts

developing from them are known to be polyvalent, is chiefly the result of the studies of Bryce,[23] Danchakoff, and Maximow. The study of hematopoiesis in birds and reptiles has shown that the granulo- and erythropoiesis, both developing from a common source, proceed independently under different environmental conditions (Danchakoff[24]). By this means the form and the finer structure of cells, derived from the polyvalent mesenchymal cells were thought to be the result of definite chemicophysical influences. The mesenchymal cells and the hemocytoblasts do not escape, under the conditions of the present experiments, the influence of the chemicophysical agents. The granulocytoblasts as usual develop from hemocytoblasts outside the vessels, and the hemocytoblasts derived from the mesenchymal cells partly differentiate into small lymphocytes, as is normally the case. The experiments confirm the statement that the differentiation is dependent upon the environmental conditions. The proliferation, however, seems to be influenced by specific stimuli which also enable a partial deviation of the normal differentiation.

The stimuli for the proliferation of the cells in the loose mesenchyme, which are strong in the early periods of embryonic development, decline in later stages, and the proliferation of these cells stops in certain parts of the organism and proceeds very slowly in others. These cells may be regarded as being "in a period of rest from which they may be aroused by special substances" (Loeb[25]). After grafting, the presence in the circulation of specific products of metabolism from the grafted spleen cells produces a strong irritation in the mesenchymal cells. The mesenchymal cells respond to this irritation by growth,

[23] Bryce, T. H., The Histology of the Blood of the Larva of *Lepidosiren paradoxa.* Part I. Structure of the Resting and Dividing Corpuscles, *Tr. Roy. Soc. Edinburgh,* 1904, xli, 291; Part II. Hæmatogenesis, *ibid.*, 1905, xli, 435.

[24] Danchakoff, V., Untersuchungen über die Entwickelung des Blutes und Bindegewebes bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo und der Dottersack als blutbildendes Organ, *Anat. Hefte,* 1908, xxxvii, 471; Ueber die Blutbildung im Dottersack des Hühnchens, *Verhandl. anat. Ges., Anat. Anz.,* 1908, xxxii, Suppl., 72; Ueber die Entwicklung des Knochenmarks bei den Vögeln und über dessen Veranderungen bei Blutentziehungen und Ernährungsstörungen, *Arch. mikr. Anat.,* 1909, lxxiv, 855.

[25] Loeb, J., The Stimulation of Growth, *Science,* 1915, xli, 704

multiplication, and differentiation, which in this case is into granulocytoblasts and granulocytes.

Loeb[26] attributes the stimulation of growth to the influence of certain substances. Gudernatsch[27] has shown that the feeding of thyroid substance has a stimulating effect upon the growth of the limbs of the tadpole. In the latter case the thyroid substance merely accelerates the normal rate of proliferation of embryonic cells.

The sudden and general proliferation of the mesenchymal cells and their further differentiation in the embryo body after grafts on the allantois includes also cell groups which would normally remain indefinitely in a period of rest. This sudden proliferation is accompanied by a differentiation, which would also not have taken place here. In this respect the results of my experiments correspond closely to those of Loeb in connection with the regeneration in *Bryophyllum calycinum.* Only the wound stimulus is absent in the experiments with grafting, and the proliferation and differentiation of the mesenchymal cells in the embryo body are probably stimulated by the appearance in the embryonic tissues of specific substances, involving the growth of certain kinds of cells. This power of stimulating the growth at will and of directing the differentiation of the mesenchymal cells may offer further suggestions in the study of hematopoiesis.

For our partial problem the deviation of the normal differentiation of the polyvalent stem cells in the thymus into another line seems to me to be definite proof of the true lymphatic nature both of the stem cells and of the small cortical cells.

The Small Thymus Cell Is a True Small Lymphocyte. It May Differentiate into a Granular Lymphatic Cell and into a Plasma Cell.

The various potentialities of differentiation in a polyvalent mother cell may under definite conditions be used for the identification of the daughter cells. The further differentiation of the daughter cells may also serve as criteria for cell identification.

[26] Loeb, J., Rules and Mechanism of Inhibition and Correlation in the Regeneration of *Bryophyllum calycinum, Bot. Gaz.*, 1915, lx, 249.

[27] Gudernatsch, J. F., Feeding Experiments on Tadpoles. II. A Further Contribution to the Knowledge of Organs with Internal Secretion, *Am. J. Anat.*, 1914, xv, 431.

The tissue of birds, particularly the loose connective tissue, contains characteristic wandering cells, which show in their cytoplasm numerous small spherical eosinophilic granules. These cells develop at the expense of the small lymphocytes in later stages of embryonic life (Danchakoff), and their presence in the connective tissue of the adult animal has been described by Solucha.[28] The present study has shown that this process of differentiation of specific granular lymphocytes also takes place physiologically in the normal thymus glands. Fig. 6 represents a small group of such cells in the loose connective tissue and Fig. 7 a group of similar cells in the thymus. The cell marked x shows the gradual accumulation of small eosinophilic granules.

A development of the small thymus cells into another line of differentiation, namely, into plasma cells, may be induced by administration to the animals of small doses of x-ray. Schaffer described analogous changes of small lymphocytes into plasma cells as occurring during the physiological involution of the thymus. Numerous small thymus cells differentiate under these conditions into plasma cells. Fig. 8 shows a group of developing plasma cells situated in the reticulum. It is easy to follow the gradual development of a plasma cell from a small thymus cell (S. Lmc.). The cytoplasm becomes more abundant and basophilic and the nucleus undergoes characteristic changes (x). The chromatin condenses into larger blocks which usually adhere to the membrane. This change leads finally to the formation of a typical *Radkern*.

Normal thymus glands, excised before the treatment of the animals, show that the occasional differentiation of the small thymus cells into plasma cells is due to the specific influence of small doses of x-rays.

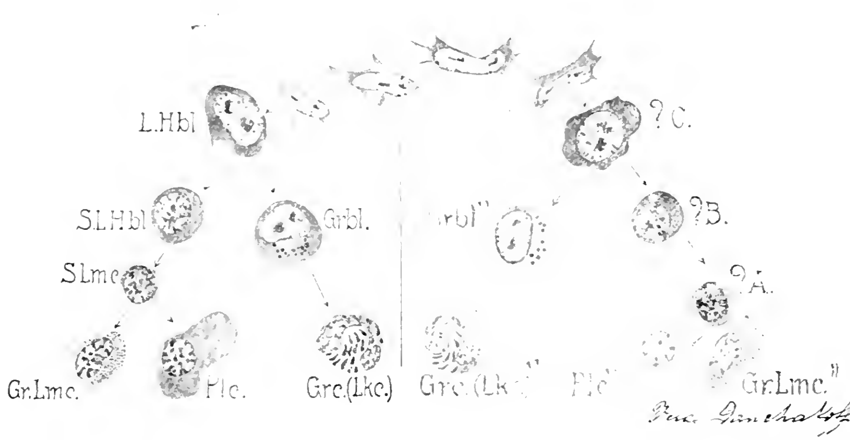
The experimental and physiological transformation of the small thymus cells and their mother cells into products peculiar only to the group of lymphoid elements thus causes the small thymus cells and their mother cells to be included in this group.

It is important in this connection, that the various differentiations described above are confined under the conditions cited, both to the thymus cells and their stem cells, as well as to the true small lymphocytes and the lymphoid hemocytoblasts found in other parts of the organism. The differentiation of the hemocytoblasts into granulo-

[28] Solucha, N. P. The Different Kinds of Cells in the Connective Tissue. Dissertation, St. Petersburg, 1908.

cytoblasts and granulocytes has already been mentioned. The physiological differentiation of the small lymphocytes into granular lymphocytes, and the experimental differentiation of the small lymphocytes into plasma cells is equally general.

The principle of differentiation of cells as a criterion for cell identification was considered in the present paper in relation to the small cortical cells of the thymus. The same principle may serve as a criterion for the identification of other cells, the morphological structure of which does not offer sufficiently definite data for their identification.



TEXT-FIG. 1. Scheme for some of the differentiation products of the loose mesenchyme.

SUMMARY.

The main problem which has been considered in this paper is the identification of the small thymus cells. Cells resembling ?A infiltrate the cortical layer of the thymus. Whether these cells are true lymph cells, or whether they merely represent a disguised epithelial cell, could not be solved either by purely histological or by histo- and embryogenetic studies.

Text-fig. 1 gives a scheme for the differentiation of the small thymus cells and the small lymphocytes, which led to a conception of the lymphoid nature of the small thymus cells. On the left of the figure is shown the origin and the normal differentiation of the small

lymphocytes and their mother cells in birds. The source of the lymphoid cells is the loose mesenchyme. The small lymphocytes (S. Lmc.), normally situated in the spleen, in the connective tissue, and in the bone marrow, appear as differentiation products of the lymphoid hemocytoblasts (L. Hbl.), especially when the latter multiply intensely in a limited space, become smaller (S. L. Hbl.), and finally change their morphological structure. The small lymphocytes may themselves under definite conditions undergo further differentiation and develop into plasma cells (Plc.) and into granular lymphocytes (Gr. Lmc.), specific for birds. The mother cells of the small lymphocytes, the lymphoid hemocytoblasts, on the other hand, differentiate in granulopoietic organs (spleen, bone marrow) into granulocytoblasts (Grbl.) and granulocytes (Grc. (Lkc.)).

The histogenetic study of cell ?A shows that its nearest stem cells are represented by the cells ?B and ?C (page 93). The striking similarity in the structure of cell ?A with that of the small lymphocyte (on the left of the text-figure), as well as the analogy of the process of its differentiation, at the expense of cells ?B and ?C, with the normal origin of the small lymphocytes, has led many investigators to accept the mesenchymal origin of the small thymus cells. According to this view, cell ?A becomes a true small lymphocyte. In addition to this is the observation of an invasion of the epithelial thymus anlage by mesenchymal elements.

According to the transformation theory cell ?A has become a disguised epithelial cell. The same was admitted for their stem cells ?B and ?C. As mentioned above, many characteristic morphological features of cell structure were shown to be not as essential as previously admitted. Nerve cells and epithelial and connective tissue cells may all assume a spherical shape in a liquid medium. How then are we to regard the small thymus cell ?A ?

If the mother cell of the thymus cell ?A is really a lymphoid hemocytoblast, it must possess the differentiation potentialities of a true lymphoid hemocytoblast; it must have the faculty of differentiating not only into small lymphocytes but also into granulocytoblasts (Grbl.") and further into granulocytes (Grc. Lkc."). If the small thymus cell ?A is a small lymphocyte it must under definite conditions be able to differentiate in the directions characteristic of true small

lymphocytes: namely, into plasma cells (Plc.") and into granular lymphocytes (Gr. Lmc."). The existence of these possibilities for differentiation of the small thymus cells ?A and their stem cells ?C have been experimentally proved by this study.

It has been shown that the mother cell ?C may under certain conditions proliferate intensely and differentiate into granulocytoblasts and granular leukocytes. This is probably due to the stimulating action of certain metabolic products of the grafted spleen cells.

It has been also shown that the small thymus cell ?A may differentiate physiologically into a granular lymphocyte, and under administration of x-ray into a plasma cell.

All the changes observed on the small thymus cells and their mother cells apply under equal conditions to the lymphoid hemocytoblasts and to the true small lymphocytes in the regions of the organism where these cells are present.

EXPLANATION OF PLATES.

All the figures were drawn with the camera at stage level, with Zeiss Apochromat 2 mm. oil immersion objective, and No. 12 compensating ocular.

The abbreviations are the same for all the figures. Fb., fibroblast; Grbl., granulocytoblast (myelocyte); Grc., granulocyte (leukocyte); Gr. Lmc., granular lymphocyte; L. Hbl., lymphoid hemocytoblast (large lymphocyte); Msc., mesenchyme cell; Rtcc., reticulum cells in the thymus; S. L. Hbl., small lymphoid hemocytoblast; S. Lmc., small lymphocyte; S. Lmc.', mitosis of small lymphocyte; x, a transition cell between two stages of differentiation; Plc., plasma cell.

Plate 8.

Fig. 1. Cortical layer of a normal thymus from a 12 day embryo; intensive development of small lymphocytes at the expense of the lymphoid hemocytoblasts, L. Hbl., with intermediate stages of small lymphoid hemocytoblasts, S. L. Hbl., and transition stages, x.

Fig. 2. Cells showing the gradual differentiation of a small lymphocyte, *f*, at the expense of a large lymphoid hemocytoblast, *a*, with intermediate stages, *b*, *c*, *d*, *e*.

Fig. 3. Mitotic figures; *a* and *b* of a lymphoid hemocytoblast, *c* of a small lymphocyte.

Plate 9.

Fig. 4. Cortical layer of a thymus of a 12 day embryo after grafting. Differentiation of the lymphoid hemocytoblast into two various directions; into

small lymphocytes (S. Lmc.), into granulocytoblasts (Grbl.), and into granulocytes (Grc.).

FIG. 5. Cells showing the gradual differentiation of a granular leukocyte, *f*, at the expense of a lymphoid hemocytoblast, *a*, with intermediate stages *b, c, d, e*.

PLATE 10.

FIG. 6. A group of granular lymph cells in the loose connective tissue.

FIG. 7. A group of granular lymph cells in a normal thymus.

FIG. 8. A group of developing plasma cells in a thymus after administration of x-ray.

10b

A CONTRIBUTION TO THE CHEMOTHERAPY OF TUBERCULOSIS.

First Experimental Report.*

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Plates 11 to 15.

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I.

Since the epoch-making discovery of Ehrlich and Hata in the treatment of syphilis, various investigators have attempted to apply chemotherapy to tuberculosis. The results of my experiments, up to the present, will be given in this communication.

In 1890 Koch reported before the Tenth International Medical Congress that the influence of telepine oil, aromatic substances, β-naphthylamine, *p*-toluidine, xylidine, dyes such as fuchsine, gentian violet, methylene blue, quinoline yellow, auramin, the steam of mercury, and chemical substances containing silver or gold, had been tested upon the growth of tubercle bacilli. Of these, potassium auricyanide was found to have remarkable germicidal power. In a dilution of 1 : 2,000,000 it kills the tubercle bacillus *in vitro*, but is powerless *in vivo*. Although the cyanides of various metals have certain specific affinities for the tubercle bacillus, yet potassium auricyanide seems to change into an inert substance by the action of the organic cells even before it reaches the tubercular lesion or the bacillus itself. While I was working on experiments to prove this point, Fischer reported that the excretions of the epidermis of a tubercular patient, as well as tuberculin, may prevent 0.5 per cent solution of potassium ferrocyanide and 1 per cent solution of ferric chloride from combining to form Berlin blue. This shows that the excretions may combine with one of the two chemical substances before they combine with each other. On the other hand, Finkler, von Linden, Meissen, Strauss, and others, reported that copper exerts an influence on the tubercle bacillus, and I have also proved this by experiments.

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*The original paper in Japanese was read before the Alumni Meeting of the Kitasato Institute for Infectious Diseases, Tokyo, Apr. 4, 1915.

I prepared a compound of copper and cyanide, both of which are known to exert an influence on the tubercular lesion. This compound may have an injurious effect upon the organic body unless it is treated chemically before being introduced into the body. The chemical compound, which is the subject of this article, has been prepared with a specific treatment so as to lessen the lesional reaction. With this product I have made experiments in the chemotherapy of tuberculosis.

Preliminary Experiments.

Guinea pigs were employed in the preliminary experiments. 2 mg. of the human type of the tubercle bacillus were introduced under the skin on the left side of the chest, and observations recorded of the effect of the preparation upon the duration of life, body weight, and macroscopical changes of various organs, as well as of the microscopical changes and the number of tubercle bacilli. Of six guinea pigs injected with tubercle bacilli, three were kept as controls and the remaining three were treated with the chemical preparation.

0.5 cc. (0.1 of a lethal dose) of the solution of potassium cyanide, diluted 1 : 10,000, was given subcutaneously every other day. All the experimental animals died after gradual loss of body weight, while the controls remained comparatively healthy. The macroscopic and microscopic changes in the treated animals exceeded those of the controls. In a dilution of 1 : 1,000, however, this chemical preparation will inhibit the growth of the tubercle bacillus *in vitro*.

The potassium cyanide was treated with a specific manipulation in order to prevent the formation of free hydrogen cyanide and the subsequent chemical changes into potassium and ammonium formate, which process is accelerated by heating. The preparation thus treated has been found not to decompose even after a lapse of 6 months. The chemical substance thus prepared is called Liquid A. The lethal dose of this solution is 0.025 gm. per kilo of body weight in rabbits, and 0.005 gm. per 100 gm. of body weight in guinea pigs. In this case Control Animal 1 died of general miliary tuberculosis 11 weeks after the injection of the tubercle bacilli, preceded by loss of body weight and dyspnea. The treated animals, on the contrary,

began to increase in body weight and were livelier in their movements, and I therefore killed them 1 week after the death of the control animal. In the controls large numbers of bacilli were found in the lungs, liver, and spleen, while in Treated Animals 2 and 3, which received the hypodermic injection of the liquid, these organs contained few or even practically no bacilli. Moreover, the infection site of the treated animals consisted of an inactive cicatrix, as observed under the microscope, while that of the control was in a progressive state. In Treated Animal 4, which received the injection of the liquid at an interval of 5 weeks from the time of infection, the infection site was not healed, and the left inguinal gland showed a cheesy degeneration. Microscopical examination showed the tuberculosis still to be progressive, yet a remarkable elimination of the bacilli and an increase in body weight were also observed. From these results it may be inferred that tubercle-infected animals that receive hypodermic injections of Liquid A will outlive the control animals, and present a marked arrest of the pathological lesions. In order to determine the curative effect of Liquid A, the experiment was repeated with the results shown in Table I.

Advancing tuberculosis indicates active inflammation around the lesion where the nodules are present, dilatation of the blood vessels, and infiltration of the leukocytes and small round cells. Sometimes the nodules contain giant cells, accompanied by marked cheesy degeneration and with small production of epithelial cells.

Suspended tuberculosis indicates that the lesions contain giant cells and present cheesy degeneration, but show little inflammation about the edges of the nodules or in the surrounding tissues, while a vigorous growth of epithelial cells is taking place, accompanied by infiltration of small round cells.

Arrested tuberculosis indicates that the lesion is devoid of giant cells or cheesy degeneration, while the nodules themselves are changed into connective tissue, perhaps surrounded by muscle tissue, with only slight infiltration of small round cells.

Control Animal 5 died of general miliary tuberculosis in 12 weeks (Table II); Treated Animals 7 and 11 lived in apparent health for 10 months, when they were killed for examination. No. 6 died of cold in 21 weeks; No. 8 of a bite in 22 weeks; No. 9 of hemorrhage into the abdominal cavity in 22 weeks; No. 10 of an external wound in 27 weeks; No. 13 of ascites in 22 weeks. Microscopical examination showed that only in No. 11 were progressive pathological changes present, while the remaining seven treated animals harbored a small number of

TABLE I.

Curative Effect of Liquid A.

No. of animal.	Weight.	Duration of life.*	Change in body weight.	Macroscopic changes.	Microscopic changes.†	No. of bacilli.
Control.						
	gm.	wks.	gm.			
1	245	11 +	−50	*Infection site:* ulceration. *Lymph glands:* swelling, cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
Hypodermic injection of 0.5 cc. of Liquid A every other day.						
2	275	12 ⊖	+27	*Infection site:* cicatrization. *Lymph glands:* swelling size of pea. *Lungs, liver, and spleen:* 1 or 2 nodules in each.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	− − −
3	270	12 ⊖	+180	Same as No. 2.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	− − +
4	250	12 ⊖	+200	*Infection site:* ulceration. *Lymph glands:* swelling, central cheesy degeneration. *Lungs, liver, and spleen:* 1 or 2 nodules in each.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+ − +

*⊖, killed; +, died; +++, very many bacilli; ++, many bacilli; +, more than 10 in the whole surface of the preparation; −, negative on microscopical examination.

† p. t., progressive tuberculosis; m. t., miliary tuberculosis; s. t., suspended tuberculosis; c. t., cured tuberculosis.

TABLE II.

Experiments with Liquid A.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Control.						
	gm.	*wks.*	*gm.*			
5	550	12 +	−150	*Infection site:* ulceration, pustulation. *Lungs:* cheesy degeneration. *Liver:* m. t. *Spleen:* general cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
Hypodermic injection of 0.5 cc. of Liquid A every other day.						
6	450	21 +	+150	*Infection site:* ulceration. *Lymphatic glands:* swelling size of pea. *Lungs:* brown pigment infiltrated. *Liver:* 1 or 2 nodules. *Spleen:* few nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ − +
7	450	43 ⊕	+305	*Infection site:* cured, cicatrization. *Lymphatic glands:* large as tip of small finger, central cheesy degeneration. *Lungs:* no change. *Liver:* indurated. *Spleen:* no change.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ − +
8	350	22 + (External wounds.)	+150	Same as No. 7, but no swelling in the lymphatic glands.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +

TABLE II—*Concluded.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
9	450	26 +	+100	Same as No. 8.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
10	500	39 + (Intestinal catarrh.)	+250	Same as No. 8.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
11	450	43 ⊕	−120	*Infection site:* cured, cicatrization. *Lymphatic glands:* no swelling. *Lungs:* m. t. *Liver and spleen:* no change.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* " "	++ ++ ++
12	500	26 + (External wound.)	+200	*Infection site:* cured, cicatrization. *Lymphatic glands:* no swelling. *Lungs, liver, and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
13	450	36 +	−+	*Infection site:* cured, cicatrization. *Ascites:* about 50 cc. *Lungs:* no change. *Liver:* indurated. *Spleen:* normal size, m. t.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +

the bacilli only. In other words, the pathological changes were arrested. These results agree with those of the original experiment in which a marked improvement in the course of the tuberculosis was noted.

Experimental Ophthalmic Tuberculosis in the Rabbit.

For the purpose of determining the influence of Liquid A upon tubercular lesions, experiments with guinea pigs are inconvenient, for the animals must either be allowed to die or they must be killed before any changes can be detected. Hence I injected the human type of tubercle bacilli into the aqueous chamber of the eye of the rabbit, in which situation the changes could be observed freely.

TABLE III.

Experimental Ophthalmic Tuberculosis in Rabbits Treated with Liquid A.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
			Control.			
	gm.	*wks.*	*gm.*			
1	2,550	9 ⊕	−200	General ophthalmic tuberculosis. *Lungs:* metastatic m. t.	*Eyeball:* p. t. *Lungs:* " "	+++ +++
			Injection of 5 cc. of Liquid A every other day.			
2	2,100	5 + (Abortion.)	+150	*Right iris:* 4 nodules. *Lungs:* no nodules.	*Eyeball:* s. t. *Lungs:* normal.	++ —
3	2,100	7 + (Ileus.)	+250	Same as No. 2.	*Eyeball:* s. t. *Lungs:* pneumonia by foreign body.	++ —
4	1,700	9 ⊕	+200	Same as No. 2.	*Eyeball:* s. t. *Lungs:* " "	++ +
			Control.			
5	1,000	56 ⊕	+1,600	General ophthalmic tuberculosis. *Lungs:* m. t.	*Eyeball:* p. t. *Lungs:* " "	+++ +++
			Injection of 7 cc. every other day, first 2 times; 2.0 cc. every other day since then.			
6	2,000	56 ⊕	+1,250	*Iris:* cured, cicatrization. *Lungs:* normal.	*Eyeball:* c. t. *Lungs:* " "	— —

Table III indicates that Control Animal 1 developed first tubercular nodules on the surface of the iris. These gradually increased in number and volume and contained a large number of bacilli, and finally underwent cheesy degeneration leading to severe miliary tuberculosis. On the other hand, Treated Animal 2 presented five nodules at the edge of the pupil, which, however, soon disappeared. The four nodules appearing on the surface of the iris remained stationary and were confirmed by autopsy. Treated Animal 3 developed an irregular pear-shaped pupil in consequence of an adhesion of the iris and cicatrization. The tubercular nodules were surrounded by newly formed connective tissue, and a calcareous deposit took place within the cavity that had been formed by the cheesy degeneration; a few bacilli only were present. The lungs contained one or two nodules in which tubercle bacilli were not found. Treated Animal 4 presented about the same number and size of nodules present before the injection of the liquid. They contained small numbers of bacilli.

In the control animal the infection was progressive, and in the treated animals it was suspended. It therefore occurred to me that if the treatment should be continued long enough the disease might be completely healed. With this idea in mind I made another experiment with two guinea pigs. One of them, No. 5, was kept as a control. This animal developed general ophthalmic tuberculosis and miliary tuberculosis of the lung. The other, No. 6, which received continuous treatment with the liquid was completely cured, the only residue being a cicatrization at the point of inoculation. No nodules developed after a period of 6 months, and none during a succeeding period of 6 months.

Liquid A produced a congestion around the ophthalmic lesions which sometimes amounted to hemorrhage within the lesions, while the nodules became stained with the coagulated blood. Moreover, the internal pressure of the eye increased and the eyeball seemed to be pushed outwards. These phenomena disappeared with the discontinuance of the injection of the preparation and reappeared upon its renewal. On the contrary, the healthy eye seemed to be unaffected by the injection. Hence I infer that the effects are evidence of the influence of Liquid A upon the tubercular lesion. Whether the congestion and the bleeding are due to the action of the freed toxin or endotoxin of the tubercle bacilli, or whether they result from some irritant property of the liquid itself reacting on the toxin of the tubercle bacilli, has not yet been determined. It is clear, how-

ever, that the cyanide radical has a special chemical affinity for the toxin; for, as pointed out previously, the cutaneous excretion of a tubercular patient, as well as tuberculin, has an inhibiting action upon the chemical composition of Berlin blue. This fact, discovered by Fischer, is called the color reaction of the cutaneous excretion of tubercular patients. From this I conceive that congestion and bleeding of the tubercular lesions may be due to some such interaction, and the theory is supported by the fact that in a dilution of 1 : 1,000,000 to 1 : 2,000,000 potassium auricyanide will kill tubercle bacilli *in vitro*. Koch has stated that no other chemical substances produce a similar result.

First Comparative Treatment with Various Preparations.

The literature gives little information regarding the chemotherapy of tuberculosis. Schröder, Haupt, and others investigated the influence of tuberculin upon experimental ophthalmic tuberculosis, but report only negative results. Schröder referred to the fact that similar negative results have been observed by Alexander, Baumgarten, and their followers. In my experiments the disease seems to have been suspended in Treated Animals 2, 3, and 4, while in No. 6 it was completely arrested, leaving only the cicatrization after which there was no recurrence. These facts indicate that Liquid A may have a decided value in the treatment of tuberculosis.

I made a comparative study of the following three curative materials for tuberculosis, which are at present thought to be most promising. I employed two of my original preparations; that is, Liquid A and Liquid B. The latter contains one-half as much potassium cyanide as the former. The remaining three preparations are: (1) copper chloride, which has been suggested by Fischer, experimentally studied by von Linden, and clinically applied upon tubercular patients, especially in lupus, by Meissen and Strauss; (2) albumin-free tuberculin; (3) iodotuberculin. Unfortunately I could not obtain the iodotuberculin as offered by Kapsenberg, Sternberg, Bauer, and Murschhauser, and I therefore prepared a substitute by allowing 20 per cent of iodine to act upon old tuberculin (Merck). The preparation did not react to starch.

TABLE IV.

First Comparative Treatment of Tuberculosis with Various Preparations.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
			Controls.			
	gm.	wks.	gm.			
14	605	8 +	−125	*Infection site:* ulceration. *Lymphatic glands:* swelling size of pea. *Ascites:* about 5 cc. *Lungs:* m. t. *Liver and spleen:* general cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
15	773	9 +	−173	*Infection site:* pustulation. *Lymphatic glands:* swelling and cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver and spleen:* m. t.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
16	552	8 + (External wounds.)	−25	*Infection site:* ulceration. *Lymphatic glands:* swelling large as tip of small finger. *Lungs and liver:* m. t. (Spleen was eaten by rats.)	*Lungs:* p. t. *Liver:* " "	+++ +++
17	580	9 +	−38	Same as No. 15.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

TABLE IV—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
18	577	11 +	−119	*Infection site:* cured, cicatrization. *Lymphatic glands:* swelling size of pea. *Lungs:* cheesy pneumonia. *Liver and spleen:* general cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
19	400	9 +	−92	*Infection site:* ulceration. *Lymph glands:* swelling and cheesy degeneration. Right pleural cavity contained about 5 cc. of serous excretion. *Lungs, liver, and spleen:* same as No. 18.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
20	720	6 +	−94	Same as No. 14. (Ascites about 25 cc.)	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
21	660	6 +	−45	Same as No. 15.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
22	480	11 +	−93	Same as No. 14.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
23	570	10 +	−+	Same as No. 14.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

TABLE IV—*Continued.*

Copper Chloride.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
The same as von Linden's 6th experiment.						
	gm.	*wks.*	*gm.*			
28	575	17 ⊖	+81	*Infection site:* drainage. *Lymph glands:* swelling size of pea. *Lungs:* several nodules big as rice grains. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
29	465	13 +	−52	Not clear.	Not clear.	Not clear.
The same as von Linden's 4th experiment.						
30	625	4 +	−90 (Mortification of site.)	*Infection site:* ulceration. *Lymph glands:* swelling, with central large cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
The same as von Linden's 5th experiment.						
31	500	12 +	−18	*Infection site:* cured, cicatrization. *Lymph glands:* no swelling. *Lungs:* m. t. *Liver and spleen:* both slightly swollen.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	++ + +
32	515	8 +	−4	Same as No. 31.	*Lungs:* s. t. *Liver:* p. t. *Spleen:* s. t.	+ ++ +

TABLE IV—*Continued.*

Albumin-Free Tuberculin.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Doses the same as those used by Schröder.						
	gm.	*wks.*	*gm.*			
33	640	11 +	−108	*Infection site:* ulceration; flowing pustulation. *Lymph glands:* swelling size of pea. *Lungs:* cheesy pneumonia. *Liver and spleen:* cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
34	535	4 + (Anaphylaxis.)	−55	Same as No. 33, with about 25 cc. ascites.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
35	560	6 +	−22	Same as No. 34.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
36	335	7 + (Hemorrhage into abdominal cavity.)	+113	*Infection site:* cured, cicatrization. *Lymph glands:* central cheesy degeneration size of pea. *Lungs:* 1 or 2 nodules. *Liver:* no change. *Spleen:* m. t.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
37	360	10 +	+67	*Infection site:* ulceration. *Lymph glands:* swelling size of pea. *Lungs:* cheesy pneumonia. *Liver:* no change. *Spleen:* cheesy degeneration.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* p. t.	+++ ++ +++

TABLE IV—*Continued.*

Iodotuberculin.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Doses the same as those used by Schröder.						
	gm.	*wks.*	*gm.*			
38	540	11 + (Intestinal strangulation.)	−93	*Infection site:* ulceration; small intestine cohered to abdominal walls. *Lungs:* cheesy pneumonia. *Liver and spleen:* no nodules.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* p. t.	+ + ++
39	680	7 +	−66	*Infection site:* cured, cicatrization. *Lungs:* cheesy pneumonia. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
40	480	8 +	−83	Not clear.	*Lungs:* not clear. *Liver:* " " *Spleen:* " "	+++ +++ +++
41	560	17 ⊕	+204	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
42	565	17 ⊕	+107	*Infection site:* pustulation. *Lymph glands:* swelling size of pea. *Lungs:* 1 or 2 nodules. *Liver:* no nodules. *Spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +

TABLE IV—*Continued.*

Liquid A.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Injection of 1 cc. of Liquid A every other day.						
	gm.	*wks.*	*gm.*			
43	460	11 + (External wound.)	+72	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of pea, central cheesy degeneration. *Lungs, liver, and spleen:* no marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
44	565	7 +	−82	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of pea. *Ascites:* about 5 cc. *Lungs:* m. t. *Liver and spleen:* 1 or 2 nodules.	*Lungs* p. t. *Liver:* s. t. *Spleen:* " "	++ + +
45	577	7 + (Bitten by a dog.)	−123	*Infection site:* cicatrization, surroundings being infiltrated. *Lymph glands:* swelling size of pea. *Lungs:* a few nodules. *Liver and spleen:* no marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
46	538	12 +	+78	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of rice grains. *Lungs:* a few nodules. *Liver and spleen:* no marked changes.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* p. t.	++ + ++

TABLE IV—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
47	460	7 + (Pneumonia.)	−45	*Infection site:* ulceration. *Lymph glands:* swelling size of rice grains. *Lungs:* a few nodules. *Liver and spleen:* no marked changes.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* p. t.	++ + +
48	540	12 + (Ascites.)	+92	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of rice grains. *Ascites:* about 20 cc. *Lungs:* pigment infiltration. *Liver and spleen:* no marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
49	460	12 +	+68	*Infection site:* cured by cicatrization. *Lymph glands:* swelling size of pea. *Lungs:* a few nodules. *Liver and spleen:* no marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
50	650	9 + (Pleurisy.)	−62	*Infection site:* ulceration. *Lungs:* cheesy pneumonia. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
51	460	13 + (External wound.)	+50	No marked changes except about 10 cc. of pleural excretion.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
52	430	17 ⊕	+176	No marked changes.	Same as No. 51.	+ + +

TABLE IV—*Concluded.*

Liquid B.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Hypodermic injection of 2 cc. of Liquid B every other day.						
	gm.	*wks.*	*gm.*			
53	490	9 +	+27	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of rice grains. *Lungs:* m. t. *Liver and spleen:* no marked changes.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ ++
54	680	13 + (Pneumonia.)	+35	Same as No. 53.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ +++ +++
55	618	13 +	+117	*Infection site:* cured, cicatrization. *Lungs, liver, and spleen:* a few nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
56	640	12 +	−+	Same as No. 55.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
57	498	16 + (Pneumonia.)	+77	Same as No. 53	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ +++ +++

Controls.

From Table IV it will be seen that Control Animals 14 and 16, 15, 17, and 19, 20 and 21, 18 and 22, and 23 died of general miliary tuberculosis in the 8th, 9th, 6th, 11th, and 10th weeks after inoculation, respectively. Microscopical examination showed progressive tuberculosis and many bacilli in all.

The time in which death occurs in experimental tuberculosis, as reported by various authors, differs according to the site of injection, the number of bacilli, and the toxicity of different strains of bacilli. Spengler reports death in 11 weeks; Schröder in from 18 to 75 days; von Linden in from 8 to 11 weeks; and Haupt in from 7 to 3 months and from 5 to 7 months, respectively. Haupt injected from 1 : 10,000 to 1 : 100,000 mg. of bacilli into the femoral muscle. The strain which I used seems to have been of medium grade of toxicity. Of the five animals treated with copper chloride, No. 28 gained in body weight and showed no objective changes in appearance until the end of the 17th week, when it was killed for examination. The macroscopic changes were conspicuous and the microscopic ones were progressive, and associated with the presence of many bacilli. No. 29 was missing. No. 30 was dead in 4 weeks; it presented extensive necrosis at the site of injection of the bacilli, and contained a great many bacilli. Nos. 31 and 32 died at the end of 12 and 8 weeks, respectively, and showed only slight macroscopic changes and small numbers of bacilli. Microscopic examination of both showed them to be in the suspended stage. Thus my tests agree with those of von Linden in bringing about improvement in experimental tuberculosis by the use of copper chloride.

Albumin-Free Tuberculin.

The albumin-free tuberculin was applied from the 4th week of inoculation in five animals. No. 34 died of anaphylactic shock. No. 37 died in 10 weeks of miliary tuberculosis, and microscopical examination showed it to be in the progressive stage. No. 33 died in 11 weeks, and microscopic examination showed numerous bacilli and progressive tuberculosis. No. 35 died in 6 weeks, and in appearance resembled No. 34. No. 36 gained in body weight and showed only slight macroscopic changes and a small number of bacilli. Microscopic examination indicated that the animal was in the suspended stage at the time that death occurred, in the 7th week, from hemorrhage into the abdominal cavity. Thus the results coincide with the view of Koch, Virchow, and Ziegler, who held that tuberculin and its derivatives tend to increase the resistance of the individual against tuberculosis and thus to improve the general condition, although without action directly on the bacilli.

Iodotuberculin.

Iodotuberculin was given hypodermically from the 4th week of infection. Animal 38 died of intestinal strangulation in consequence of cicatrization which occurred after the intestinal lesion had been cured. Small numbers of the bacilli were found in the lesion. The liver seemed to be in the suspended stage, but the lungs and the spleen appeared to be uninfluenced. No. 39 died in the 7th week. It had lost body weight, and on microscopic examination was found to be in the progressive stage with a great many bacilli. No. 40 had been kept for 8

weeks when it was lost. No. 41 gained in body weight and was killed for examination in the 17th week. It presented no conspicuous macroscopic changes, and microscopic examination showed it to be in the suspended stage, with a few bacilli only. No. 42 gained gradually in body weight. It was killed in the 17th week and showed slight macroscopic changes with few bacilli, and under the microscope proved to be in the suspended stage. Thus the iodotuberculin which I prepared yielded good results, although it may not have been identical with that prepared by Kapsenberg, Sternberg, Bauer, and Murschhauser.

Liquid A.

Liquid A was given from the 4th week after inoculation. Animal 43 gained in weight until it died from an external wound in the 11th week. It showed slight macroscopic changes with few bacilli, and microscopically was found to be in the suspended stage. No. 44 died in 7 weeks. It had progressive lesions in the lungs, but suspended ones in the liver and spleen with few bacilli. No. 45 was killed accidentally in the 7th week. It was in the suspended stage with few bacilli and showed slight macroscopic changes. Nos. 46 and 47 died in the 12th and 7th weeks, respectively. In both the liver was in the suspended stage with few bacilli, and the lungs and spleen were in the progressive stage with many bacilli. Nos. 48 and 49 both died in 12 weeks. They had both gained in body weight, had slight macroscopic changes, and microscopically were found to be in the suspended stage with few bacilli. No. 50 died of pleurisy in 9 weeks. It showed conspicuous macroscopic changes with many bacilli and was in the progressive stage. No. 51 died from an external wound. No. 52 gained in body weight and appeared healthy until it was killed for examination in 17 weeks. Macroscopically it was found to have slight changes, and microscopically was in the suspended stage.

Liquid B.

Liquid B was given to five animals. Nos. 53, 54, and 57 died in 9, 13, and 16 weeks, respectively. They showed conspicuous macroscopic changes, and were in the progressive stage with many bacilli. Nos. 55 and 56 died in 13 and 12 weeks, respectively. They both showed slight macroscopic changes and were in the suspended stage with few bacilli.

The results of the comparative study of the various preparations show that copper chloride and albumin-free tuberculin each brought about complete suspension in 1, and half suspension in 1 of the 5 experimental animals; iodotuberculin brought about full suspension in 2, and half suspension in 1 of the 5 animals; while Liquid A brought about complete suspension in 6, and half suspension in 3 of 10 animals, and Liquid B full suspension in 2 of the 5. Therefore, judged

according to efficiency, these preparations stand in the following order: copper chloride and albumin-free tuberculin are least efficient; Liquid B and iodotuberculin are more efficient; Liquid A is the most efficient. Yet Liquid A did not completely heal the tubercular lesions.

Second Comparative Treatment with Various Preparations.

Proceeding from the fact that Liquid A was more efficient than Liquid B, I prepared another solution which contained more potassium cyanide than the previous preparation, in the hope of obtaining a more efficient substance. The 1:1,000 aqueous solution of this substance was called Liquid C. Again, I prepared a double salt of copper and potassium cyanide. The aqueous solution diluted 1:2,000 was prepared and treated in the same manner as Liquid A, and called Liquid D. The lethal dose of this Liquid D is 0.001 gm. per 100 gm. of body weight for the guinea pig, and for the mouse 1 : 200,000, or more accurately 1:10,000 of the body weight. Hence an aqueous solution (1:2,000) of the original solution was prepared, and 1 cc. per kilo of body weight was injected hypodermically into guinea pigs in which experimental tuberculosis had been induced. Some of the treated animals died sooner than the controls and presented severer lesions both macroscopically and microscopically; in others the reverse was the case. The comparative study of Liquids C and D is shown in Table V.

Controls.

Control Animals 58, 59, and 60 gradually lost weight and died in 5 to 8 weeks. They showed marked macroscopic changes, and under the microscope were found to be in the progressive stage with large numbers of bacilli; Nos. 74 and 75 lived until they were killed for examination in the 22nd week. They showed no marked macroscopic changes. Under the microscope in No. 74 the lung was found to be in the progressive stage; the liver and spleen were in the suspended stage with only a few tubercle bacilli. No. 75 was also in the progressive stage, and showed a few or moderate number of bacilli.

The difference in the results may be due to the fact that when dilutions of 1 : 10,000 or 1 : 100,000 mg. of tubercle bacilli were given under the skin of the right femoral muscle of guinea pigs, as Haupt

TABLE V.

Second Comparative Treatment of Tuberculosis with Various Preparations.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Controls.						
	gm.	*wks.*	*gm.*			
58	647	6 +	−90	*Infection site:* infiltration. *Lymph glands:* swelling size of pea. *Ascites:* about 20cc. *Lungs:* cheesy pneumonia. *Liver and spleen:* no marked changes.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
59	587	8 +	−97	*Infection site:* pustulation. *Lymph glands:* swelling size of pea; thickening, cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver, spleen:* cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
60	450	5 +	−14	Same as No. 59.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
74	667	22 ⊕	Not clear because of pregnancy.	*Infection site:* drained pustulation. *Lymph glands:* swelling size of rice grains. *Lungs:* pigment infiltration; a few nodules. *Liver, spleen:* m. t.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* " "	+ + +
75	705	22 ⊕	Same as No. 74.	Same as No. 74.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +

TABLE V—*Continued.*

Liquid C.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Hypodermic injection of 1 cc. of Liquid C every 3 weeks.						
	gm.	*wks.*	*gm.*			
61	630	22 ⊕	+70	*Infection site:* cured, cicatrization. *Lymph glands:* swelling size of rice grains. *Lungs:* a few nodules. *Liver and spleen:* no marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + −
62	690	22 ⊕	+110	Same as No. 61 except a few nodules in liver and spleen.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + −
63	620	22 +	+90	Same as No. 62.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + −
76	622	22 ⊕	Not clear because of pregnancy.	Same as No. 62.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + −
77	755	13 +	Same as No. 76.	Same as No. 62.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + −

Liquid D.

Hypodermic injection of 1.5 cc. of Liquid D every 5 weeks.

64	709	21 +	+61	*Infection site:* cured, cicatrization. *Ascites:* about 20 cc. *Lungs:* pigment infiltration, and 1 or 2 nodules. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +
65	625	22 +	+110	Same as No. 64.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ ++ ++

TABLE V—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Hypodermic injection of 1 cc. of Liquid D every 5 weeks.						
	gm.	*wks.*	*gm.*			
66	612	22 ⊕	+216	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	– – –
67	597	29 + (Intestinal catarrh.)	+173	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	+ + –
68	467	5 + (Intestinal catarrh.)	+–	*Infection site:* ulceration. *Lungs:* 1 or 2 pigment infiltrations. *Liver and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	– + ++
78	727	22 ⊕	Not clear because of pregnancy.	Same as No. 68.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + –
79	520	15 +	Same as No. 78.	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	+ + –
80	595	15 + (Inflammation of subcutaneous fat tissue.)	Same as No. 78.	Same as No. 68.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + –
81	410	22 ⊕	Same as No. 78.	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	+ – –

TABLE V—*Concluded.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Hypodermic injection of 0.5 cc. of Liquid D every 3 weeks.						
	gm.	*wks.*	*gm.*			
69	510	19 +	+175	*Infection site:* cured, cicatrization. *Lymph glands:* big as tip of small finger. *Lungs:* 1 or 2 nodules. *Liver:* no nodules. *Spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ – –
70	532	18 + (Inflammation of the subcutaneous fat tissue.)	+54	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
71	490	20 +	+100	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
Hypodermic injection of 0.25 cc. of Liquid D every 3 weeks.						
72	547	13 + (Intestinal catarrh.)	+178	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
73	416	16 +	+169	Same as No. 69.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ – –

also reports, the animals died in from 7 to 3 and from 5 to 7 months, and therefore the date of the death of the experimental animals varied according to the site of the infection and the number and toxicity of the strain of the bacilli in question. Hypodermic injection of the bacilli does not always kill guinea pigs. Again, the duration of life as well as the microscopic findings varies according to the resistance that each animal displays towards the injection. Therefore, any representative result can be attained only by as many experiments as will yield a correct statistical approximation. In my two recent experiments, unfortunately, the guinea pigs died too soon of miliary tuberculosis to agree with the prognosis in human cases. I therefore introduced the bacilli into the veins of the treated animals. The bacillary strain was obtained through the courtesy of Dr. Shiga, and had been proven to have strong toxicity. 0.05 mg. of the strain was given intravenously. In my previous experiment subcutaneous injection of the bacilli was employed. The results, therefore, seem to be relative and not decisive.

Liquid C.

Of the five animals that had been treated with Liquid C, Nos. 63 and 77 died in 22 and 13 weeks, respectively. The remaining three animals, Nos. 61, 62, and 76, appeared healthy until the 22nd week, when they were killed for examination. They showed slight macroscopic changes with very few bacilli, and under the microscope were found to be in the suspended stage.

Liquid D.

Animals 64 and 65 received hypodermic injections of 1.5 cc. of Liquid D once every 5 weeks. The former died in 21 weeks, the latter in 22 weeks. They had both gained in body weight, but showed severe macroscopic changes, and were in the progressive stage with a great number or moderate number of bacilli. Both were worse than the controls.

Of the seven animals that have been treated with 1 cc. of Liquid D, Animals 67 and 68 died in 29 and 5 weeks of intestinal catarrh, and Nos. 79 and 80 in 15 weeks of inflammation of the subcutaneous fat tissue. Nos. 66, 78, and 81 appeared healthy until 22 weeks, when they were killed for examination. Nos. 66, 67, 79, and 81 showed no macroscopic changes, while all the others showed slight microscopic changes. Nos. 66, 67, 79, and 81 were found to be cured of tuberculosis; the rest were in the suspended stage. No. 66 was free from bacilli, and the remaining six animals were found to have few or no bacilli in their organs.

Three animals received 0.5 cc. of Liquid D once every 3 weeks. No. 69 died

in 19 weeks, No. 70 died of inflammation of the subcutaneous fat tissue in 18 weeks, and No. 71 died in 20 weeks. They had all increased in body weight and showed slight macroscopic changes and suspended lesions with only a few bacilli.

Nos. 72 and 73 received 0.25 cc. of Liquid D once every 3 weeks. The former died of intestinal catarrh in 13 weeks, and the latter died in 16 weeks. They had both gained in body weight, and similar macroscopic as well as microscopic changes occurred, as noted in the preceding animals which had received 0.5 cc. of Liquid D once every 3 weeks. From these data it may be concluded that the animals which had been treated with 1 cc. of Liquid D given once every 5 weeks showed most improvement; Liquid C is less effective. Less improvement was obtained in proportion as less of Liquid D was administered. It is especially notable that the changes in the animals which received 1.5 cc. of Liquid D were more severe than in the control animals and showed more bacilli than the latter.

To sum up the results obtained in my preliminary experiments, it may be stated that subcutaneous injection of 1 cc. of Liquid D once every 5 weeks produced prolongation of life, increase of body weight, improvement in the macroscopic changes, and a cure, as indicated by the microscopic examination. Other preparations produced some improvement in the tubercular lesions, but they were efficacious only in suspending the changes determined by microscopic examination. The dose and the best manner of administering the remedy can only be determined by further study.

The preliminary experiments described above were conducted in the Surgical Institute of the Kyoto Imperial University, in the service of Professor Ito. I desire here to express my thanks to him and to Assistant Professor Osaki for their kindness in affording me the means and direction that were necessary for carrying on my experiments.

II.

Since February, 1914, I have conducted experiments with Liquid D in experimental tuberculosis at the Kitasato Institute. I undertook to explain the inconstant effects observed; namely, that while pathological processes are made to subside in some, other animals remain indifferent to the action of the preparation. By the aid of the microscope I observed the fact that copper potassium cyanide consists of three different forms of crystals, and I have, by employing a special method, succeeded in separating the different forms. I experimented with each of the three substances and ascertained that only

one of them is effective against tuberculosis, while the remaining two have an injurious effect upon the pathological processes. Since that time I have employed only the effective substance. I have tried to see what effect this substance will have on the healthy animal.

First Animal Experiments.

Two guinea pigs were chosen for the purpose; one received five injections intravenously of 1 cc. of Liquid D per kilo of body weight once every other week, while the other received the same dose at the same interval, but subcutaneously. Both gained in body weight and showed no changes.

Liquid D will inhibit the growth of all tubercle bacilli in the common glycerin broth culture, when it is mixed in the proportion of 1 : 10,000; a solution of 1 : 15,000 will inhibit the growth of the bacilli in a blood serum agar culture. Strains fast to the drug could not be obtained, though the attempt was made during a period of 5 months.

In the following experiments I employed the human type of a certain virulent strain given me by Dr. Shiga. An emulsion was prepared and 0.05 mg. of the bacilli introduced into the femoral vein. The injection of Liquid D was begun on the 4th day after inoculation. The results of the experiment are shown in Table VI.

Controls.

From Table VI it will be seen that all five control animals died in from 4 to 14 weeks. They showed marked macroscopic changes, and were in the progressive stage of tuberculosis, as seen under the microscope, with many tubercle bacilli.

Iodotuberculin.

Five animals were treated with iodotuberculin, of which two died, one in 3, the other in 4 weeks, before the efficiency of the preparation could be seen. No. 88 died in 12 weeks. It had slight macroscopic changes and was in the suspended stage with only a few bacilli. No. 90 died in 17 weeks. It showed slight macroscopic changes, and the lung was in the progressive stage; the liver and spleen were in the suspended stage. Many bacilli were found in the lungs, but few in the liver and spleen. No. 91 died in 18 weeks. It had slight macroscopic changes; microscopically the lung was found to be in the progressive stage with few bacilli.

TABLE VI.

First Animal Experiments.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Controls.						
	gm.	*wks.*	*gm.*			
82	330	4 +	−30	Highly emaciated. *Lymph glands:* swollen and cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver:* 1 or 2 nodules. *Spleen:* cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
83	240	14 +	+20	Same as No. 82.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ ++
84	280	9 +	−+	Same as No. 82.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
85	355	8 +	−10	Same as No. 82.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +++
86	165	4 +	+30	Same as No. 82.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
Iodotuberculin. Doses the same as those used by Schröder.						
87	340	3 +	−50	*Lymph glands:* swollen and cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

TABLE VI—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
88	260	12 +	+30	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
89	240	4 +	−20	*Lymph glands:* swelling size of pea. *Lungs:* m. t. *Liver:* " " *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
90	460	17 +	+10	*Lymph glands:* swelling size of rice grains. *Lungs:* 1 or 2 nodules. *Liver and spleen:* no marked changes.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* " "	++ + +
91	210	18 +	+160	Same as No. 90.	*Lungs:* p. t. *Liver:* s. t. *Spleen:* " "	+ + +

Liquid D.

Intravenous injection of 1 cc. of Liquid D every 3 weeks.

92	290	23 ⊕	+120	*Lymph glands:* big as tip of small finger. *Lungs:* 1 or 2 nodules. *Liver:* 1 " 2 " *Spleen:* 2 or 3 grayish white protuberant nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ − +
93	400	23 ⊕	+40	*Lymph glands:* swelling size of pea. *Lungs:* 3 vacuoles and 1 or 2 nodules. *Liver:* 1 or 2 nodules. *Spleen:* 1 " 2 "	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
94	240	18 +	−30	Same as No. 92.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ − +

TABLE VI—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Intravenous injection of 1 cc. of Liquid D every other week.						
	gm.	*wks.*	*gm.*			
95	240	23 ⊕	+400	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	– – –
96	280	23 ⊕	+250	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	– 4 2
97	310	23 ⊕	+20	*Lymph glands:* swelling size of pea. *Lungs:* no marked changes. *Liver:* 1 or 2 nodules. *Spleen:* 1 " 2 "	*Lungs:* c. t. *Liver:* s. t. *Spleen:* " "	– + +
Intravenous injection of 1 cc. of Liquid D every week.						
98	240	23 ⊕	+170	*Lymph glands:* swelling size of pea. *Lungs:* 1 or 2 nodules. *Liver and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
99	260	9 +	+150	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
100	240	15 +	+70	Not clear.	Not clear.	Not clear.
101	350	14 +	+10	Not clear.	Not clear.	Not clear.
102	280	13 +	+10	Not clear.	Not clear.	Not clear.

TABLE VI—*Concluded.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Hypodermic injection of 1 cc. of Liquid D every week.						
	gm.	*wks.*	*gm.*			
103	450	12 +	0	*Lymph glands:* swelling size of rice grains. *Lungs:* 1 or 2 nodules. *Liver and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
104	520	13 +	−80	Same as No. 103.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
105	375	11 +	+150	*Lymph glands:* swollen, cheesy degeneration. *Lungs:* m. t. *Lungs and spleen:* m. t.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +++
106	340	23 ⊕	+350	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	− − −
107	420	8 +	−80	*Lymph glands:* swollen. *Lungs:* m. t. *Liver:* " " *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

Liquid D.

Three animals were treated with an intravenous injection of 1 cc. of Liquid D once every 3 weeks. No. 94 died in 18 weeks, and Nos. 92 and 93 were killed in the 23rd week. Macroscopically they showed slight changes, while microscopically they were in the suspended stage of tuberculosis. Only a few bacilli were discovered in some organs.

Of the three animals that received intravenously 1 cc. once every other week, Nos. 95 and 96 had been healthy until they were killed for examination in the 23rd week. They bore no observable macroscopic changes and showed traces of healed tuberculosis under the microscope. The former showed no bacilli, while the latter showed four in the liver and two in the spleen. No. 97 was killed in the 23rd week. It also showed slight macroscopic changes. Under the microscope

the lungs showed traces of healed tuberculosis, without any bacilli, while the liver and spleen showed suspended tuberculosis with few bacilli.

Of the five animals that received intravenously 1 cc. of Liquid D weekly, No. 98 was healthy until it was killed for examination in the 23rd week, while No. 99 died in the 9th week. Macroscopically the former had slight changes and the latter practically none. Microscopically both were in the suspended stage with few bacilli. Nos. 100, 101, and 102 died in the 15th, 14th, and 13th weeks, respectively. Unfortunately they were either badly preserved or overlooked before a thorough examination was made.

Of the five animals that received weekly hypodermically 1 cc. of Liquid D, Nos. 103 and 104 died in the 12th and 13th weeks, respectively. They both showed slight macroscopic changes, and microscopically were in the suspended stage with only few bacilli. No. 106 had been healthy until it was killed for examination in the 23rd week. It exhibited scarcely any macroscopic changes, and under the microscope showed traces of healed tuberculosis with no bacilli. Nos. 105 and 107 died in the 11th and 8th weeks, respectively. They showed remarkable macroscopic changes, and under the microscope were found to be in the progressive stage with numerous bacilli.

The first animal experiment proved that the intravenous injection of 1 cc. of Liquid D per kilo of body weight every other week gave the most satisfactory results, while the intravenous injection of 1 cc. once every 3 weeks, and either intravenous or hypodermic application of the same amount weekly, are inferior to the former. From these results I conclude that the best therapeutic dose is 0.05 of the lethal dose.

These experiments emphasized the fact that the grayish white nodules that seem to be miliary tuberculosis are merely connective tissue that has been made to protrude in consequence of the contraction of the fibrous tissue, and also that bacilli are sometimes present even in the surrounding region of the cheesy degeneration and in the giant cells.

Second Animal Experiments.

0.05 mg. of the human type of tubercle bacilli was emulsified and introduced intravenously into guinea pigs, which were kept for 25 days in the laboratory without previous treatment. At the expiration of this time they were given an intravenous injection of 1 cc. of Liquid C per kilo of body weight every other week. The whole

number of the experimental animals thus obtained was forty-six, eight of which were kept as controls.[1]

Controls.

From Table VII it will be seen that the eight control animals died in the 5th to the 16th weeks. They exhibited remarkable macroscopic changes, and under the microscope were seen to be in the progressive stage. No. 114 showed few bacilli, but the remaining seven had many.

Liquid D.

Treated Animals 116, 117, 118, 119, 120, 121, 122, 123, 124, and 125 had gained in body weight. Nos. 126, 127, 128 129, 130, 131, 132, 133, 134, and 135 had gained in weight and appeared healthy until killed for examination in the 22nd week. Nos. 127, 130, 131, and 132 showed no noteworthy changes, while Nos. 126, 128, 129, 133, and 135 showed a few grayish white nodules. On microscopic examination Nos. 127, 129, 130, 131, 132, and 133 showed traces of healed tuberculosis, while Nos. 126, 128, 134, and 135 had suspended lesions. One or two bacilli were sometimes found in the whole surface of the slide, but as a rule no bacilli were present. No. 128, which was killed on the 3rd day after an injection of Liquid D, showed only one bacillus in the lung preparation; all the pathological lesions exhibited the beginning of a remarkably active reaction. Nos. 136, 137, 138, 139, 140, 141, 142, 143, 144, and 145 died in the 11th to the 17th week; some gained in weight while others lost. On macroscopic examination Nos. 137, 138, 139, 140, and 141 exhibited no remarkable changes; Nos. 136, 142, 143, 144, and 145 showed only slight changes. On microscopic examination Nos. 137, 139, 140, and 141 showed healed tubercular lesions; Nos. 136, 138, 142, 143, 144, and 145 showed suspended ones. The liver of No. 145 showed small numbers of bacilli. Of the remaining animals, however, some harbored only one and at most seven bacilli per slide. Nos. 146 and 149 died in 11 and 14 weeks, respectively. On macroscopic examination only slight changes were visible, while microscopically the animals were found to be in the suspended stage with sometimes few and sometimes a larger number of bacilli. Animals 147, 148, 150, and 151 died in the 8th to the 16th weeks. Marked macro-

[1] This experiment was carried on during the time in which Dr. Kitasato left the Imperial Institute for the Study of Infectious Diseases and established the Kitasato Institute for Infectious Diseases. Owing to the lack of a proper animal house, the same care could not be taken of the animals, and hence they suffered somewhat from lack of regular food, and some developed diarrhea in consequence of the cold. The percentage of duration of life in this second experiment cannot therefore be established exactly.

TABLE VII.

Second Animal Experiments.

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
Controls.						
	gm.	*wks.*	*gm.*			
108	300	13 +	0	*Lymph glands:* swollen, cheesy degeneration. *Lungs:* cheesy pneumonia. *Liver and spleen:* cheesy pneumonia.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ ++
109	300	16 +	−20	*Lymph glands:* swelling size of pea. *Lungs:* cheesy pneumonia. *Liver:* no marked changes. *Spleen:* cheesy degeneration.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ +++ +
110	330	11 +	−20	*Lungs:* 1 or 2 nodules. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+ ++ ++
111	290	12 +	−30	Same as No. 110.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
112	290	5 +	−30	Same as No. 110.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ + ++
113	280	10 +	−20	Same as No. 110.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +
114	280	12 +	+70	*Lungs:* 1 or 2 nodules. *Liver:* several nodules. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+ + +
115	290	10 +	−30	Same as No. 110.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

TABLE VII—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight after treatment.
Intravenous injection of 1 cc. of Liquid D every other week.			
	gm.		*gm.*
116	430	Healthy.	+260
117	260	"	+ 50
118	310	"	+130
119	370	"	+ 80
120	280	"	+180
121	270	"	+ 70
122	280	"	+ 60
123	320	"	+130
124	380	"	+130
125	220	"	+ 40

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
126	330	22 ⊕	+140	*Lymph glands:* big as tip of small finger. *Lungs:* 1 or 2 nodules. *Liver:* 1 " 2 " *Spleen:* 1 " 2 "	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	2 — 1
127	300	Same as No. 126.	+210	No marked change except central cheesy degeneration size of pea in lymph glands.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	1 — —
128	310	Same as No. 126.	+59	Marked hyperemia of internal organs. *Lungs, liver, and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	1 — —
129	360	Same as No. 126.	+100	*Lungs, liver, and spleen:* several grayish white protuberant nodules.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	— — 2
130	260	Same as No. 126.	+310	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	— 1 1

TABLE VII—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
131	350	Same as No. 126.	+190	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	— — —
132	310	Same as No. 126.	+290	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	1 — —
133	280	Same as No. 126.	+100	Same as No. 129.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	1 — —
134	310	Same as No. 126.	+180	*Lymph glands:* swelling size of pea. *Lungs:* inflamed. *Liver and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	— — —
135	350	Same as No. 126.	+40	*Lymph glands:* big as tip of small finger, central cheesy degeneration. *Lungs:* 1 or 2 nodules. *Liver:* no marked changes. *Spleen:* cheesy degeneration.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	— — —
136	250	13 +	−20	*Lymph glands:* swollen, large central cheesy degeneration. *Lungs:* inflamed. *Liver and spleen:* 1 or 2 nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	3 2 1
137	230	16 +	−+	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	1 1 1

TABLE VII—*Continued.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
138	290	14 +	−50	No marked changes.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	3 — —
139	300	16 +	−+	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	— — —
140	310	17 +	+90	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	2 1 6
141	300	15 +	+15	No marked changes.	*Lungs:* c. t. *Liver:* " " *Spleen:* " "	2 — —
142	260	15 +	−+	Same as No. 136.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	7 5 3
143	300	16 +	−+	Same as No. 136.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	1 — 2
144	280	15 +	−50	Same as No. 136.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	3 — —
145	310	11 +	−50	Same as No. 136.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	7 + 7
146	300	11 +	−50	*Lymph glands:* swelling size of pea. *Lungs:* grayish white nodules. *Liver:* grayish white nodules. *Spleen:* grayish white nodules.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + + + + +

TABLE VII—*Concluded.*

No. of animal.	Weight.	Duration of life.	Change in body weight.	Macroscopic changes.	Microscopic changes.	No. of bacilli.
	gm.	*wks.*	*gm.*			
147	320	11 +	−40	*Lungs:* cheesy pneumonia. *Liver:* m. t. *Spleen:* " "	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
148	280	8 +	−30	*Lymph glands:* swollen, cheesy degeneration. *Lungs:* m. t. *Liver and spleen:* m. t.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ + +
149	270	14 +	−+	Same as No. 146.	*Lungs:* s. t. *Liver:* " " *Spleen:* " "	+ + +
150	280	14 +	−30	Same as No. 147.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	++ ++ +
151	280	16 +	−+	Same as No. 147.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++
152	250	22* +	+10	*Lymph glands:* swollen. *Lungs:* small nodules scattered between several grayish white protuberant nodules. *Liver:* the same. *Spleen:* the same.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +
153	460	22* +	+50	No marked changes.	*Lungs:* p. t. *Liver:* " " *Spleen:* " "	+++ +++ +++

* These two animals died of the reaction arising from a sudden increase of the dose, which was doubled and which led to a marked development of the pathological processes.

scopic changes were present and progressive tuberculosis with a large number of tubercle bacilli. In the last four animals the action of Liquid D was not yet complete.

Nos. 152 and 153 were given twice the dose of the liquid in the 22nd week and died of the reaction 4 days later. On macroscopic examination the former

showed slight changes and the latter none. On microscopic examination the tissues surrounding the lesion were in a state of great congestion and masses of lymphatic cells as well as leukocytes were present. The lesions were surrounded by a new growth of granulation and connective tissue, but the centers showed cheesy degeneration.

In spite of the various obstacles to which the last series of animals had been subjected, the following results were obtained. Of 38 animals, 20 had remained apparently healthy for 22 weeks, when 10 were killed for examination, of which 6 exhibited indications of healed tubercular lesions, and 4 were in the suspended stage. Of the remaining 18 animals, 2 died in consequence of the reaction caused by the sudden increase of the dose of the preparation, and hence may be deducted from the total number. Of the 16 left, 4 showed traces of healed tuberculosis, 8 were in the suspended stage, and the remaining 4 in the progressive stage similar to the controls.

SUMMARY.

Judging from the macroscopic and microscopic study of the animals treated with the liquid, its action upon the tubercular lesions seems to be about as follows: The effect of a single injection upon the lesions is either negative or inconspicuous. But after repeated injections of the preparation the congestion and leukocytic infiltration about the lesions are markedly decreased, the cheesy material resulting from degeneration of the lesions and other degeneration products are in process of absorption, and young connective tissue is being actively produced in the periphery. While these changes are taking place the number of the bacilli is also being reduced until finally they can no longer be detected on microscopic examination.

Hence it appears that while the preparation may lack bactericidal action *in vivo* powerful enough to destroy all the bacilli at one injection, yet repeated infusions may nevertheless bring about the destruction of all the bacilli and the modification of the tubercular lesion into that of the suspended stage or even into the healed condition. Whether, therefore, the preparation brings about these results directly by killing the bacilli or indirectly by favoring the healing processes of the body, nevertheless it has power to inhibit the growth of or annihilate entirely the bacilli *in vivo*.

The experiments reported leave no doubt that Liquid D is capable of bringing about the healing of experimental tubercular lesions; but thus far that most important problem in chemotherapeutics, namely, the extent of the cure produced, has not been solved. The experiments indicate that sterility of the tissues as far as microscopic examinations go has been secured; but microscopic examination is not after all an absolute test of sterility.

In order to test this point, emulsions were made of the lungs, liver, spleen, and other organs of Treated Animals 134 and 135 (Table VII), and they were inoculated into the abdominal cavity of guinea pigs. Some of the animals receiving the emulsion developed tuberculosis, and therefore absolute sterility of the treated animals had not been obtained in these instances.

The problem of the destination and distribution of the preparation in the body of the treated animal, as well as its action against the tubercle bacilli, lesions, and the tubercular organs of the infected guinea pigs, is now being studied further with results to be reported at some future time.

BIBLIOGRAPHY.

Bauer, J., and Murschhauser, H., Zur Chemotherapie der Tuberkulose, *Berl. klin. Woch.*, 1912, xlix, 1888.

Fischer, C., Eine Farbenreaktion des Hautsekretes über tuberkulösen Lungenabschnitten, *Münch. med. Woch.*, 1912, xlix, 1813.

Haupt, H., Untersuchungen über die therapeutische Wirkung des Tuberkulins gegen die Tuberkulose des Meerschweinchens und Kaninchens, *Beitr. Klin. Tuberk.*, 1911, xxiii, 471.

Kapsenberg, G., Zur Chemotherapie der Tuberkulose, *Berl. klin. Woch.*, 1912, xlix, 879.

Koch, R., Ueber bacteriologische Forschung, *Deutsch. med. Woch.*, 1890, xvi, 756.

von Linden, G., Beiträge zur Chemotherapie der Tuberkulose. Die Ergebnisse des Finklerschen Heilverfahrens bei der Impftuberkulose des Meerschweines, *Beitr. Klin. Tuberk.*, 1911, xxiii, 201.

Meissen, E., Meine Erfahrungen bei Lungentuberkulose mit Jodmethylenblau und Kupferpräparaten, *Beitr. Klin. Tuberk.*, 1911, xxiii, 215.

Schröder, G., Ueber die Wirkungen des Alttuberkulins; experimentelle Studie, *Beitr. Klin. Tuberk.*, 1906, vi, 397.

Spengler, C., Ueber die Behandlung tuberculöser Meerschweinchen mit Originaltuberkulin, *Z. Hyg. u. Infektionskrankh.*, 1897, xxvi, 323.

Sternberg, A., Zur Frage der "rationellen" Chemotherapie, *Berl. klin. Woch.*, 1912, xlix, 1606.

Strauss, A., Meine Erfahrungen mit Jodmethylenblau und Kupferpräparaten bei äusserer Tuberkulose, speziell bei Lupus, *Beitr. Klin. Tuberk.*, 1911, xxiii, 223.

EXPLANATION OF PLATES.

Plate 11.

Fig. 1. Control guinea pig (No. 111). All the nodules present a grayish yellow color and are transparent. They are more or less cirrhotic. The spleen is greatly swollen.

Plate 12.

Fig. 2. Treated guinea pig (No. 113). All the nodules present a grayish white color. In the liver they are cirrhotic. The spleen remains small.

Plate 13.

Fig. 3. Control guinea pig (No. 111). Tuberculous nodule in the liver. Leitz oc. 1, obj. 7.

a, focus of caseous degeneration; *b*, infiltrated layer; *c*, giant cells.

Fig. 4. Fibrous nodule in the lung of a treated guinea pig (No. 123). Leitz oc. 3, obj. 3.

a, nodule; *b*, parenchyma of the lung; *c*, calcareous deposit; *d*, glandular change of the bronchioles caused by chronic interstitial inflammation.

Plate 14.

Fig. 5. Fibrous nodule of the liver of a treated guinea pig (No. 123). Leitz oc. 1, obj. 7.

a, nodule; *b*, lobuli hepatici; *c*, intrahepatic bile ducts; *d*, blood vessel.

Fig 6. Fibrous nodule of the spleen of a treated guinea pig (No. 123). Leitz oc. 1, obj. 7.

a, nodule; *b*, follicle; *c*, sinus venosus.

Plate 15.

Fig. 7. Fibrous nodule of the inguinal glands of a treated guinea pig (No. 123). Leitz oc. 1, obj. 7.

a, nodule; *b*, follicle.

The above paper exceeds the limit of size set by the *Journal*. The reason for publishing it in spite of its length is found in the present importance of the subject of the specific treatment of tuberculosis to which the paper is a contribution.—The Editor.

A CONTRIBUTION TO THE CHEMOTHERAPY OF TUBERCULOSIS.

First Clinical Report.*

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(Received for publication, December 1, 1915.)

In the previous paper I have presented data on the specific chemotherapeutic treatment of experimental tuberculosis with my preparation of copper cyanide, called Liquid D. I desire now to present some of the results of the application of the treatment to human cases of tuberculosis. The treatment was first applied on September 26, 1914, at the Imperial Institute for the Study of Infectious Diseases, at which time Professor Kitasato was still its director. When he left the Institute on November 6 of the same year and established the Kitasato Institute for Infectious Diseases I followed him to the new institution where the treatment has been continued. In all, sixty-three treated cases are included in this report. The results have been tabulated as follows:

TABLE I.

The Treatment of Tubercular Patients with Liquid D.

		Total No. treated.	Cured.	Improved.	Died.	Treatment suspended.	Under treatment.
Non-active tuberculosis	1st stage.	19	13	2	—	1	3
	2nd "	6	3	3	—	—	—
	3rd "	4	1	3	—	—	—
Active tuberculosis	1st "	7	4	2	—	—	1
	2nd "	11	3	5	—	1	2
	3rd "	8	—	3	3	—	2
Surgical tuberculosis......		8	1	4	—	2	1
Total.............		63	25	22	3	4	9

*The original paper in Japanese was read before the Alumni Meeting of the Kitasato Institute for Infectious Diseases, Tokyo, Apr. 4, 1915.

The term "cured" is applied to the cases that gained in body weight, showed temperatures below 37°C., no or nearly no physical signs, and bacillus-free sputum, though before treatment the sputum abounded in bacilli; and the patients did not complain and could attend to their regular work. This class of patients is being examined monthly at present in order to determine the permanence of the cure. The term "improvement" is applied to those cases that are still under treatment or in which treatment has been discontinued; they have gained in body weight, and show temperatures below 37°C., while bacilli are occasionally found in the sputum, and other symptoms are still present. "Under treatment" is applied to cases which are at the beginning of the treatment and in which the influence of the preparation is not yet evident. One of the patients classed as active tuberculosis in the second stage has passed into the third stage, in consequence of lack of nourishment preceded by hemoptysis.

I shall describe briefly the effect of the preparation upon the lesions and upon the patients, before reporting the typical cases.

Influence of Liquid D upon the Lesions.

In one case of acute progressive pulmonary tuberculosis, which had been negative to the von Pirquet test, the preparation produced no effect. But in other cases of pulmonary tuberculosis, there appeared, on the day following the injection, dullness accompanied by râles; if the dullness and râles were present before treatment, the former became more distinct, and the latter increased. These changes are indicated in the chest charts (Cases 7 and 9), and they disappear or diminish on the 8th or 9th day after the injection. By repeated injections the regional reactions gradually disappear or diminish, but by increasing the dose of the preparation they reappear. With each successive disappearance of the signs physical regional phenomena also diminish, until finally they disappear altogether. The dullness at the site of the old lesions does not always disappear; sometimes it is converted into a short tympanic sound. In a few cases the dullness as well as the râles diminish or disappear immediately after the first injection. In Case 2 and in five other cases it was difficult to detect the regional sign before injection, but on the day following the injection râles and dullness appeared by which the lesions could be easily detected, as is depicted in the chart. From these reactive phenomena, as well as from the animal experiments, we have concluded that congestion occurs in a high degree about the lesion immediately after the injection of the preparation. However, in four cases in which bloody expectoration was present, the preparation produced no injurious effect upon the bleeding; and in two of the

four bleeding ceased on the day after the injection. The cases in the third stage generally show stronger reactions, which sometimes last as long as 2 weeks.

In cutaneous tuberculosis a local hyperemia and edema arise in a few hours after the injection. In one case pustulation of the lesion was observed 24 hours after injection. These reactions usually disappear in from 8 to 9 days, and at the same time the lesion diminishes greatly in size. With repetition of the dose the reactions diminish until at last they do not appear at all. Therefore, the minimum dose should first be given, and increased by 0.5 mg. each time subsequently. In this way the same degree of reaction may be obtained each time.

Five cases of tuberculosis of the lymphatic glands have been treated. One patient suffered from slight pain at the site of the lesion, but no swelling was observed. After two injections the lesion was completely absorbed. One case suffered from severe pain immediately after the injection. The gland became swollen considerably and ulcerated on the 2nd day. The remaining three cases suffered from swelling in the region of the gland, and pain on the following day, which disappeared in 7 to 8 days. In two of the three patients, four injections have led to the complete absorption of the lesion, while in the remaining case a hard nodule the size of the tip of the small finger, and feeling like cartilage, remains.

In one case of tuberculosis of the kidney (Case 3), the upper part of the left kidney was found on palpation to be swollen to the size of the fist. On pressure hyperesthesia and hematuria were observed. The latter ceased on the 2nd day, while the swelling and pain disappeared on the 7th day after injection. With the subsidence of the pain at the time of urination, the urine became markedly clearer than before treatment. Unfortunately the patient ceased to come for treatment after the second injection.

One case was suffering from vertebral caries with open suppuration accompanied by pulmonary tuberculosis in the first stage and tuberculosis of the lymphatic glands. The patient complained of oppressive feeling in the breast cavity after each injection. After twelve injections the patient was relieved of discomfort and was able to return to work.

In two cases of intestinal tuberculosis, the injection brought about a temperature of 40°C. with ten to twenty bloody mucous diarrheal stools 3 days later. These phenomena disappeared in 2 days. Three more cases of intestinal tuberculosis were subjected to the special precaution of evacuating the contents of the bowels beforehand, and thus these inconveniences were avoided.

Eighteen cases that had been receiving tuberculin and vaccine treatment according to Shiga's method showed neither local reactions nor rise of body temperature. The duration of the disease was considerably shortened. Case 6 is typical of these cases.

Influence upon the Tubercle Bacilli in the Sputum.

A bacteriological examination was made of the first sputum expectorated each morning in six cases in the second and third stages. The results have been recorded according to Gaffky's table and arranged diagrammatically in curves (Text-figs. 1 to 6).

In Case 2 a remarkable increase was observed from the day following injection. The climax was reached on the 10th day. From the 11th day, however, a gradual decrease was noted, and negative results were obtained on the 14th day. On the second injection of the preparation and afterwards, a slight increase was observed each time, but from the 10th day a decrease was noted. At present negative results are always obtained even after the injection. In Case 3 the decrease was observed immediately after the injection. Negative results were obtained in 2 weeks. The patient ceased to receive the treatment after that day, and has not reported since. In Case 5 the decrease was observed immediately after the first injection. Negative results were obtained from the 8th to the 14th day, when the second injection was given. On the day following the second injection, an increase to No. II of Gaffky's table was observed, but on the 3rd day negative results were obtained. Since that time two injections have been given with negative results on each examination. In Case 8 innumerable bacilli appeared on the day following the injection, but they decreased to Gaffky's No. II on the 14th day. The second injection again resulted in the appearance of innumerable bacilli. A remarkable decrease was observed from the 9th day, and at last on the 14th day negative results were obtained. From the day following the third injection a gradual increase was observed until the climax was reached on the 9th day. Since then a gradual decrease has been noted, and negative results were obtained on the 14th day. Each examination after that gave a negative result, although five injections have been made. Case 9 was in the third stage of pulmonary tuberculosis. On the 3rd day after the first injection a great increase in the number of bacilli was observed. From the 5th day they decreased, but on the 11th day innumerable bacilli were observed for the second time. They decreased on the following day. After the second injection a gradual increase of the bacilli was noted, and no decrease was observed. The fourth and the fifth injections had no effect. The physical phenomena grew worse, the temperature curve did not fall, and the fever reaction became permanent. No further treatment was given on account of weakness. Case 10 also belonged to the third stage. On the 3rd day after the first injection, innumerable bacilli appeared, but on the 5th day a gradual decrease was observed. On the 9th day negative results were obtained, but from the 10th to the 14th day innumerable bacilli were again observed. The results of the examination on the 13th day were negative, as the material for examination was unsatisfactory, and they are therefore not given in the chart. The second injection gave a better result, and the third resulted in a slight increase on the day following the injection

A gradual decrease was observed and a negative result finally obtained. After that four injections were always given, the dose being increased each time. The dose was then decreased slightly, and at present a negative result is nearly always obtained.

The film method of bacteriological examination of the sputum is liable to error, for the concentration of the specimen, the thickness of the film, and the material selected produce different results. The more exact antiformin treatment of the sputum is, therefore, to be preferred. As the examination of the first morning expectoration for several days in succession gives important data as to the effect of the preparation, I chose certain cases upon which these successive examinations were carried out. The results were found to correspond with the changes of the local reactions and the temperature curves. One of the phenomena that should be especially noted is that a patient in the first stage of pulmonary tuberculosis who ejected no bacilli in the first sputum on the day of the injection, often passed as many bacilli as correspond to Gaffky's Nos. II and III on the 2nd and 3rd days after the injection. To a patient in the third stage of the disease, a large dose either exceeding or the same as that given to a patient in the first or second stage results only in an intensification of the symptoms or an increase in the number of bacilli. Changes in the form of the bacilli were observed on the 2nd or 3rd day after the injection. The bacilli will then present the following three varieties: (*a*) the granules in the bacilli become indistinct and thus the interior becomes somewhat homogeneous; (*b*) the bacilli which have divided into several sections resemble streptococci; (*c*) the bacilli which have collected in large masses resemble staphylococci.

The three degenerated forms of the bacilli are often visible when cavities are present in the lung or the specimen has been overheated for staining purposes, and special precautions must therefore be taken before the examination is made. If the bacteriological examination of the patient in the first stage shows that the bacilli in the sputum are normal, the bacilli of the same case after the first injection will be degenerated forms similar to those often discovered in the sputum of patients having lung cavities. It may be conjectured that this degeneration in the form of the bacilli is produced by the injection

of the preparation. The sputum will, however, contain normal bacilli again 5 weeks after injection. The transformation gradually sets in, beginning with the 2nd or 3rd week.

Influence upon Body Temperature.

In more than twenty cases in which the body temperature ranged between 37° and 38°C. before injection, the temperature subsided completely after one or two injections and did not rise again above 37°C., as shown in the temperature curve of Case 1.

The curve of Case 4 shows that the temperature which previously ranged between 38° and 39°C. gradually fell after the first injection, and after the second did not rise above 37°C. This was a case of pulmonary tuberculosis in a woman in the third stage, with great weakness. She showed general improvement in consequence of the reduced temperature, and was therefore dismissed from the hospital. Unfortunately she had a chill the same night and her temperature rose to 40°C., due to a pneumonic attack, which subsided after complete rest, the temperature falling again to 38°C. The lesion, however, was observed to have extended considerably. The third injection brought about no improvement, and the patient died of debility. From this it will be seen that patients suffering from pulmonary tuberculosis in the third stage should be kept quiet for a period of 2 weeks after the injection. In three cases in which there had been no rise of temperature, it rose to 38–39°C. after slight exercise within 3 or 4 days after the injection. A localized pneumonic attack also occurred in these cases, but subsided after rest of about 3 days. Hence I emphasize the fact that in cases of slight pulmonary tuberculosis the patients must be kept quiet for 3 to 4 days after each injection.

Case 3 had a febrile attack on the 3rd day after the first injection (the dose being 16 mg.), in spite of having been kept quiet. After the second injection (same dose) the temperature rose only to 37.7–38°C., and the third injection (15 mg.) was not followed by fever.

Case 8 was given 15 mg. for the first injection. The temperature became normal.

Case 9, as already described, had a severe local reaction.

Case 10 was one of pulmonary tuberculosis in the third stage, the temperature reaching 39.8°C. Three injections of 16 mg. each were given, followed by a normal temperature and improvement of the physical signs. The bacilli in the sputum became considerably less. With the hope of accelerating the favorable effects 17 mg. were given for the fourth injection. There followed increase of the bacilli in the sputum and rise of temperature above 38°C. After 3 weeks' rest in bed the temperature fell to normal, after which the fifth and then the sixth injection, each consisting of 16 mg., were given. The temperature remained normal, the physical phenomena improved, and the bacilli in the sputum almost

disappeared. As a result of this experience we believe that the dose and the interval between each two injections should be governed by special precautions. Reduction of the dose to 13 mg. in cases suffering from pulmonary tuberculosis in the third stage, and in cases of spreading pulmonary tuberculosis followed by pleurisy (Case 9), produce, as a rule, no rise of temperature, but rather a reduction to normal. Drs. Otani, Okawara, and Yabe are carrying on clinical observations of various kinds in the Kitasato Institute, in order to learn the variety and nature of the reactions and the manner of their control. I have also asked more than twenty specialists in Japan to use my preparation, and reports from these sources may therefore be looked for. In three cases the temperature began to rise on the 10th day after each injection. It has not yet been determined whether the next injection in these cases should be made on the 11th day.

Influence upon Body Weight.

During the 3 days after injection, decrease of the body weight, amounting to 0.5 to 1 kilo, was observed in cases of the first or second stage. But before the next injection, or in 11 days, the weight will again rise to the point present before the injection, and usually even higher. However, with the cases showing a certain intensity of reaction, or in the third stage, the loss following the injection may not be regained. This result might perhaps be avoided, if more time were allowed between each two injections. Among the sixty-three cases which I have treated, a loss of weight has been observed in six only, while all the rest gained more or less.

Influence upon the von Pirquet Reaction.

Three cases in which the von Pirquet reaction was fading were given the preparation with the result that the regional infiltration reappeared and swelling was produced. In one case a blister was formed, which disappeared in 2 or 3 days. This phenomenon may indicate that the preparation has a certain action upon the toxin of the tubercle bacillus.

Thirteen cases that had given positive von Pirquet reactions were reexamined from 2 to 3 months after the disappearance of all clinical and bacteriological signs, and all were negative.

Influence upon the Subjective Symptoms.

A marked improvement is often produced in cases of pulmonary tuberculosis in the first or second stage, while in the third stage severe fatigue is felt for 3 or 4 days. Sometimes cases in the first or second stage also suffer from fatigue. At the same time a slight flush or night sweat may result, which disappears in 2 or 3 days.

Directions for the Use of the Preparation.

As a result of the experimental tests in animals on the toxicity of the preparation, I have come to the conclusion that 0.6 gm. may be introduced without injury into the veins of a man weighing 60 kilos. Moreover, it was found that a dose sufficient to establish complete cure in the experimental tuberculosis of the guinea pig is 0.5 mg. per 500 gm. of body weight. Hence, a curative dose of the preparation for a man seems to be 0.1 gm. The great difference in the species of animals was, of course, taken into consideration, and therefore in the beginning I gave a dose of 10 mg. to human cases.

Dr. Kojima, who had been suffering from pulmonary tuberculosis for 10 years, offered himself for the first trial of the preparation; he developed ileus and strangulation of the intestine and died before the influence of the preparation could be seen. The test proved, however, that in the dose employed the preparation was non-toxic for man.

The preparation is colorless, transparent, and of neutral reaction. If allowed to stand in the air for more than 6 hours, crystallization sets in. It must therefore be used as soon as the ampules are opened.

The preparation is highly irritating to the tissues and hence neither subcutaneous nor intramuscular injection can be employed and it must be injected exclusively into a vein. Injected into the subcutaneous tissues or muscles it causes severe pain and swelling. After injection the patient must be kept absolutely quiet in bed until the reactions disappear. As a rule, 3, 7, and 14 days are the periods during which patients in the 1st, 2nd, and 3rd stages, respectively, of pulmonary tuberculosis must keep quiet. Extreme emaciation is a contraindication to the employment of the preparation.

Rest of the organs affected by tuberculosis is also necessary; *e.g.*, the lessening of the amount as well as the choice of food in intestinal tuberculosis, the application of narcotics for the prevention of hemoptysis in pulmonary tuberculosis, the application of narcotics for the purpose of decreasing cough and limitation of speech in laryngeal tuberculosis, production of artificial pneumothorax (probably) in pleural tuberculosis, and application of splints for surgical tuberculosis.

Rest is necessary because the injection causes local congestion and

therefore bleeding may occur; but, on the other hand, the injection has been shown to increase the coagulation of the blood, and consequently hemorrhage will stop if rest is maintained. The injection should be made slowly, otherwise it may cause acceleration of the heart action and giddiness.

Dose.—The primary doses for the various stages and types of tuberculosis are as follows:

	mg.
Pulmonary tuberculosis in the 1st stage	14–16
" " " " 2nd "	12–14
" " " " 3rd "	8–12
Intestinal tuberculosis	8–12
Laryngeal "	8–12
Pleural "	8–12
Peritoneal "	8–12
Tuberculosis of lymphatic glands	15–17
" " bone	15–17

The doses for cutaneous tuberculosis should be increased each time by 0.5 mg. from the second injection. For laryngeal tuberculosis, however, decreasing by 0.5 mg. after the second injection is better. For other types of tuberculosis the problem of whether to increase or decrease, to repeat the increase and decrease, or to maintain the same dose is still to be worked out.

When the fever reaction lasts more than 2 weeks, it may often be reduced by lessening the doses by 1 mg. for the subsequent injection.

For pregnant women a little smaller dose should be given.

In intestinal tuberculosis purgatives must be given on the day previous to the injection.

An interval of a fortnight has been regularly allowed in every case. I have had no instance in which even a small amount of the preparation was injected at shorter intervals.

The preparation is affected by phenol and lysol. Therefore the skin and syringe must not be disinfected with these solutions. The instruments must be disinfected by heating, then dipping in alcohol, and washing carefully with physiological saline solution. The skin should be disinfected with tincture of iodine and alcohol.

Internal administration of apricot juice will increase the free prussic acid in the body, and it should therefore not be given with the preparation. Creosote and its derivates and potassium iodide sometimes produce fever and other ill effects.

Fastness of the Bacilli.—An emulsion of the organs of Treated Animals 134 and 135,[1] which had been receiving injections of the preparation, was introduced into the peritoneal cavity of a guinea pig. Tuberculosis of the intestine and spleen, with nodule formation, was produced. By injecting Liquid D, the nodules were made to disappear. I observed a case in which the preparation was given intravenously over twenty-one times to a patient who is now improving. These facts may show that the preparation probably brings about no fastness in the tubercle bacilli.

Brief Case Reports.

Case 1.—T. F., female, aged 18 years. No information obtained about grandparents, who died when she was a child. Her father died of beri-beri in his 40th year; mother living; three brothers and sisters, one of whom died of a disease of which she knows nothing. No tendency to tuberculosis seemed to exist. She had always been healthy and free from severe illness, except diphtheria, which she contracted in her 7th year. She suffered from a slight swelling and tenderness of the bowels in Apr., 1914. The condition, which was diagnosed by the attending physicians as peritonitis, subsided in about a month. She had been well since then until the middle of Oct., 1914, when she caught cold and suffered from fever and cough.

Diagnosis.—Pulmonary tuberculosis in the second stage; serous pleurisy on left side.

Present Illness.—Weak constitution; malnutrition; skin not dry, elasticity normal; poor in subcutaneous fatty tissue; face pale; conjunctiva anemic; larynx slightly red; no swelling in the lymphatic glands of the neck; typical pigeon chest; breathing weak on the left side; percussion sound short within the region of four fingers' breadth at the apex on the right back; left lung, dullness below the third rib; on the left frontal apex and right lateral apex râles were heard; respiration weak, lacking in the vocal vibration; bowels normal; pleural lymphatic glands, no swelling or tenderness; body weight 33.2 kilos. Von Pirquet reaction, +, +, +.

Progress.—On Nov. 10, 1914, 15 mg. of the preparation were injected intravenously. From the following day the fever subsided and the general condition improved. The bacilli correspond to Gaffky's No. II. Since then twenty injections have been given. At present the patient weighs 35.2 kilos; has a dullness over the right apex; and the sputum is free from tubercle bacilli.

Case 2.—K. K., male, aged 29 years. Parents and six brothers and sisters all living and healthy; no congenital tuberculosis. Has always been weak and

[1] Koga, G., *J. Exp. Med.* 1916, xxiv, 107.

neurasthenic, but has had no severe illness except colds. 4 years ago he swallowed a piece of metal, which was removed by operation. 2 years ago a diagnosis of tuberculosis of the right apex was made. At that time the temperature was above 37.4°C., and negative results had been obtained by bacteriological examination of the sputum. Under clinical treatment all the symptoms subsided in about 3 months. In Jan., 1914, he suffered from a febrile attack, the temperature reaching as high as 38°C., with pain in the left thorax. Toward the end of the month, the fever reached 40°C. with accompanying expectorations, cough, and night sweats. Appetite unimpaired; stools once daily.

Diagnosis.—Pulmonary tuberculosis in the second stage.

Present Illness.—Constitution medium; nourishment good; skin not wet; features anemic; cheeks slightly flushed; conjunctiva anemic; no white membrane over the tongue; neck a little long; lymphatic glands of the neck not swollen; thorax normal in form; breathing weak along the left side of the thorax; by percussion on left upper lobe, above the line corresponding to the second intercostal space, dullness is observed; on auscultation râles are heard over this area; right upper lobes, bronchial sound; at the apex of the heart and left axillary region slight friction; general respiratory sounds coarse; dullness over the region of the heart; heart normal in size; heart sounds also normal; all organs in the abdominal cavity normal; urine showed no casts or albumin. Von Pirquet reaction, ++, ++, +. Bacilli correspond to Gaffky's No. IV. Body weight 53.3 kilos. Temperature 37.4°C.

Progress.—Received intravenously 16 mg. for the first time on Feb. 26, 1915. On Feb. 28 febrile reaction reaching 38.3°C. On Mar. 3 it reached as high as 39°C. Afterwards it fell to 37.6–37.7°C. The behavior of the bacilli in the sputum is shown in the chart; after an interval of 2 weeks the second injection was given. The temperature began to fall, the highest being 37.3°C. Six more injections each at intervals of 2 weeks were given. At present the temperature is 37°C. On the left side of the intercostal space one or two râles are heard; no dullness or bronchial respiration along all the remaining regions of the lungs. The bacilli correspond to Gaffky's No. II. The patient is still receiving the injection. Body weight 53 kilos.

Case 3.—T. Y., male, aged 25 years. Both parents healthy; three brothers and sisters living; two brothers died of consumption. The patient has never been strong. 3 years ago he caught cold and suffered from cough and expectoration, and very severe night sweats and fever, which improved considerably under the use of the preparation for 4 months. From Nov., 1914, a renewed attack of fever was observed. He suffered this time from hematuria; urinated fifteen times in 24 hours. He also had a severe pain at the opening of the urethra and in the lower bowels. At that time he urinated over seventeen times daily. Appetite impaired.

Diagnosis.—Catarrh of the left apex and tuberculosis of the left kidney.

Present Illness.—Constitution and nourishment impaired; skin dry; features emaciated; conjunctiva highly anemic; pulsation 110, weak; neck long; no swollen lymphatic glands; thorax symmetrical; breathing slightly weak on the left side; on percussion over the left frontal region of the lung there were dullness down to the third rib, and bronchial respiration and metallic respiratory sounds; over the whole region of the upper lobe of the left lung there were slight râles; normal precardial dullness; anemic heart sounds; left kidney slightly tender and impalpable; bladder not tender; the bacilli corresponded to Gaffky's No. III. The tubercle bacilli in the urine were moderately numerous. Urine neutral and slightly cloudy; considerable deposits were obtained by centrifugalization; albumin in the filtered liquid; no epithelial cells visible under the microscope. Von Pirquet reaction negative. Weight 35.4 kilos. Urine 1,450 cc.; urinated fifteen times daily

Progress.—On Feb. 9, 1915, 15 mg. of the preparation were injected intravenously. On the following day the left kidney was found to be tender and as large as the fist. The urine assumed a red color, erythrocytes being present; amount 1,700 cc.; urinated fourteen times. On Feb. 11 the pain in the lower bowels and at the urethral opening at the time of urination disappeared, while the urine became somewhat limpid. The deposit obtained by centrifugalization was examined microscopically, and tubercle bacilli were demonstrated. The symptoms in the lungs showed no conspicuous changes. The bacilli in the sputum are shown in the chart. Since that time the urine increased to 2,000 gm. He left the hospital and has not reported since.

Case 4.—I. O., female, aged 19 years. Father died of stomach trouble; mother and one younger brother living. No evidence of an inherited tendency toward tuberculosis. She had been healthy without any noteworthy illness since birth. She suffered from cough and night sweats since Dec., 1914. Menstruation irregular; appetite impaired; sleeps well.

Diagnosis.—Pulmonary tuberculosis in the third stage, and on the right side tubercular pleurisy.

Present Illness.—Constitution good; nourishment impaired; features agonal; cheeks flushed; conjunctiva highly anemic; skin dry; tongue has no white membrane; pulse weak, count over 105 per minute; temperature 38.1°C.; thorax symmetrical; respiration weak on the right side; right lung shows bronchial respiration down to the fourth intercostal space; right side of the back has a short sound, and on auscultation bronchial sounds with râles; right apex measures 4 by 3 cm.; normal precardial dullness; heart normal; lower bowels somewhat distended; axilla is tender, but has no dull percussion sound; no hypertrophy of the intestinal walls or lymphatic glands occurs. Von Pirquet reaction, +, +, −. The bacilli correspond to Gaffky's No VII. Body weight 43.8 kilos.

Progress.—Received intravenously 16 mg. of the preparation on Feb. 16, 1915. The body temperature fell to 37.5°C. on Mar. 1. General condition improved. Received the second injection of 16 mg. on Mar. 3. The temperature rose to 38.1°C. and fell again to 37.1–37.2°C. on the following day; general conditions

improved markedly. She was dismissed from the hospital on the 6th day after the second injection. On that evening fever set in which rose to 39.8°C. at 9 o'clock, caused by overexercise. Since that time emaciation became conspicuous, and the temperature curve became irregular. She was again admitted to the hospital. The third injection was given on Mar. 19, and the fourth on Apr. 7, each time the doses being 16 mg. No reduction of fever was produced. Appetite poor. Died on Apr. 28.

Case 5.—M. K., male, aged 27 years. Both parents healthy. No inherited tendency to tuberculosis could be traced. He contracted measles in his 2nd year, but besides this he has had no marked sickness until 4 years ago when he suffered from an external wound and a broken right fifth rib, followed by an attack of pleurisy which yielded to medical treatment. 2 months ago hemoptysis (about 299 cc.) occurred. Since then he has had several hemorrhages. He is suffering from cough, with moderate expectoration, but no night sweats. Appetite good. Stools once daily.

Diagnosis.—Pulmonary tuberculosis in the second stage; right tubercular pleurisy.

Present Illness.—Constitution and nourishment good; conjunctiva not anemic; pulse regular, 84 per minute; no jugular lymphatic gland swelling; thorax symmetrical; breathing weak on right side; on auscultation râles were heard; at the frontal border line between the right lung and the liver an inspiratory bronchial sound was heard; on the back corresponding to the lower margin of the scapula a short friction sound; the left apex had a short sound at the region corresponding to the fossa supraclavicularis; normal precardial dullness; heart sounds also normal; no change in the bowels. Von Pirquet reaction, +, +, +. The bacilli correspond to Gaffky's No. V. Temperature 37.5°C. Weight 49.1 kilos.

Progress.—The first intravenous injection of 16 mg. of the preparation was given on Jan. 8, 1915. A night sweat occurred the same night. On the following day along the border line between the right lung and the liver numerous râles were heard, but the friction sound on the back of the right lung disappeared; during the night of Jan. 12 he suffered from pulmonary hemorrhage (about 30 cc.). Since that night until Jan. 22 he has had several hemorrhages; bloody sputum was expectorated until Jan. 29. Therefore the second injection was postponed until Feb. 2, when 16 mg. were again given intravenously. The general condition showed great improvement. The third injection (16 mg.) and the fourth (17 mg.) were given on Feb. 18 and Mar. 5, respectively. The symptoms in the lung disappeared completely, and the sputum was free from tubercle bacilli. Subjective symptoms also subsided. Body weight increased to 49.8 kilos.

Case 6.—M. J., male, aged 34 years. Father died of heart disease; mother living. Has no paternal uncles or aunts; three maternal uncles and aunts, one of whom died of carcinoma. Three brothers and sisters. His elder brother died of tuberculosis and his nephew is also suffering from it.

He has been weak since birth, but has contracted no serious disease. In Apr., 1915, he was found to be suffering from pulmonary tuberculosis. Since that

time he has been receiving tuberculin treatment. At present has no subjective symptoms.

Diagnosis.—Pulmonary tuberculosis in the second stage.

Present Illness.—Both constitution and nourishment good; skin and mucous membrane give no sign of anemia. Larynx somewhat reddened; no swelling in the jugular lymphatic glands; thorax symmetrical; breathing also symmetrical. In the left lung short percussion sound down to the third rib in front and the lower edge of the scapula on the back; these regions had a weak respiratory sound while at the apex a bronchial respiratory sound was heard; the right apex has a short sound over a radius of four fingers; normal precardial dullness; bowels normal; temperature 36.9°C. Bacilli in the sputum correspond to Gaffky's No. I. Weight 36.3 kilos.

Progress.—The first intravenous injection of 16 mg. was given on Nov. 10, 1914. The left apex had a dull percussion sound, and râles appeared. Weight on Nov. 24, 36.3 kilos. On the same day the second injection of 16 mg. was given. Weight on Nov. 26, 35.8 kilos; on Dec. 8, 36.1 kilos. On Nov. 8 he had only a very weak respiratory sound at the left apex; all other symptoms absent. On Jan. 12, 1915, he was examined, but had no symptoms. The sputum also was found to be free from bacilli. On Apr. 9 body weight was 47.5 kilos. Von Pirquet reaction negative. No physical phenomena were present in the lung. No subjective symptoms.

Case 7.—S. K., male, aged 36 years. Father and mother both died of stomach trouble at 71 and 67 years, respectively. Six brothers and sisters, one of whom died of tuberculosis; the rest are living. One maternal relative is suffering from tuberculosis.

He has never been strong. In his 7th year he contracted peritonitis, which was cured in 2 months; had beri-beri in his 20th year and ever since has been suffering from cerebral neurasthenia; 6 years ago a diagnosis of tuberculosis of the apex was made, although he had no cough or expectoration; the following year climatic and tuberculin treatment were tried; he says he is susceptible to colds; at present little expectoration. Appetite normal; stools once daily.

Diagnosis.—Pulmonary tuberculosis in the second stage.

Present Illness.—Both constitution and nourishment poor; skin and mucous membrane anemic; larynx somewhat reddened; neck long; no swelling of the jugular lymphatic glands; thorax flat; the region of the manubrium sterni sunken; breathing weak on both sides; in the upper lobes of both lungs a very weak respiratory sound is present; at the right apex there is a bronchial sound: no change is perceptible by percussion; normal precardial dullness; no anemic sounds; bowels sunken, but no change is perceptible. Von Pirquet reaction, +, +, +. Tubercle bacilli in the sputum can only be demonstrated by the use of antiformin. Weight 42.8 kilos. Temperature 36.8°C.

Progress.—The first injection of 16 mg. was given on Jan. 19, 1915. On Jan. 20 the right apex had a short sound on both sides down to the third rib, where many râles were heard. Cough and expectoration present. The bacilli in the

sputum correspond to Gaffky's No. II. The second injection of 16 mg., the third of 17 mg., and the fourth of 17 mg., were given on Feb. 2 and 16, and Mar. 2, respectively; bronchial respiratory sounds at the right apex and a few râles at the left scapular region appeared on May 7, when the fifth injection of 16 mg. was given. On May 20 the body weight was 44.5 kilos. The râles had all disappeared; only the coarse respiratory sound of the right apex remained. The injection was, therefore, suspended.

The results of five sputum examinations were negative.

Case 8.—Y. I., male, aged 36 years. Father died of stomach trouble at 59 years of age, and mother died of pneumonia in her 56th year. Has six brothers and sisters, all of whom are healthy; wife and five children living; one child died immediately after birth. No inherited tendency toward tuberculosis could be established.

He has always been healthy; suffered from enteric catarrh in his 18th year; and from typhoid fever in his 28th. In Apr., 1914, contracted laryngeal tuberculosis; in August had fever, cough, and expectoration; a tender tumor as large as the tip of the little finger appeared at the anal region; it was operated on and is still excreting pus; since then all the symptoms have been worse; emaciation setting in; night sweats slight; appetite impaired.

Diagnosis.—Pulmonary tuberculosis in the second stage; left axillary lymphatic tuberculosis and tubercular ulceration at the anal region.

Present Illness.—Constitution poor; nourishment impaired; skin yellowish brown and somewhat anemic; conjunctiva anemic; larynx slightly congested; neck short and no swelling in the lymphatic glands; thorax symmetrical; breathing symmetrical; in both lungs dullness up and down the borders of the scapula; many râles were heard down to the second intercostal space; back of the left lung general prolongation of exhalation; swelling of the lymphatic glands of the left side; the left axillary glands were swollen to about the size of a pigeon's egg; tender, but no adhesions; normal precardial dullness; bowels normal; ulceration as large as a pea at the anal region; the edges show gradual transition, but no new growth of the mucous membrane; pus is present. The bacilli in the sputum correspond to Gaffky's No. VI. Von Pirquet reaction, +, +, −. Weight 46.5 kilos. Urine free from albumin and casts.

Progress.—The first injection of 15 mg. was given on Jan. 16, 1915. Severe sweats occurred that night, but subsided in 2 days; on Jan. 24 no râles were heard; on Jan. 29 the second injection of 16 mg. was given. That night a slight pain was felt at the left axillary glands; by massaging the region the swelling disappeared; the excretion of pus from the anal lesion ceased; a slight increase of expectoration was noted; the third injection of 16 mg. was given on Feb. 12, and fever subsided completely. All the clinical symptoms had gone except the short percussion sound at the left apex. The temperature and the results of bacteriological examination of the sputum are shown in the chart. Since then the sputum was examined several times under the microscope, but negative results were obtained. Weight 48.3 kilos.

Case 9.—A. Y., female, aged 26 years. Father died of consumption; mother still living. One paternal uncle died of apoplexy; maternal uncles and aunts are all healthy; has three brothers and sisters, all of whom are healthy. Husband and two children also healthy.

The patient has been healthy since birth until Apr., 1914, when she had a hemorrhage; since then she has had five hemorrhages; case diagnosed as pulmonary tuberculosis; receiving climatic and medical treatment; appetite impaired; occasionally diarrheal stools two or three times daily, which, however, soon subsided.

Diagnosis.—Pulmonary tuberculosis in the third stage; right tubercular pleurisy.

Present Illness.—Constitution poor; nourishment impaired; skin and mucous membrane anemic; cheeks flushed; larynx slightly congested; neck long; no lymphatic glands swollen; typical pigeon chest; right lung very weak in respiratory sounds; slight dullness of frontal side of the right lung down to the fourth intercostal space; râles at apex; bronchial respiration elsewhere; slight dullness over lateral side of the right lung; râles and friction sounds also heard; few râles on lateral side of left lung at intercostal space; normal precardial dullness; no tenderness or hard nodules in the bowels. Body temperature 38.1°C. Pulse over 101. Body weight 43.7 kilos. The bacilli in the sputum correspond to Gaffky's No. VI.

Progress.—The first injection of 16 mg. was given on Feb. 9, 1915. On Feb. 14 the temperature fell to 37.6°C., when the patient was discharged from the hospital. In the evening the temperature rose to 38.2°C. The third injection (16 mg.) and the fourth (14 mg.) were given on Mar. 9 and Apr. 27, respectively, but brought about gradual rise of temperature, more bacilli in the sputum, and extension of the lesions, as shown in the chart. Emaciation gradually set in, and the body weight fell to 38.2 kilos. The injection of the preparation was therefore suspended for the time being.

Case 10.—E. T., male, aged 34 years. Father died of an indefinite disease; mother healthy. Six brothers and sisters, one of whom died while young, also of an indefinite disease. No inherited tendency toward tuberculosis.

The patient had been healthy until he was about 14 years old, when he contracted pleurisy after a cold, and then tuberculosis. The symptoms were becoming worse. In 1914 he was operated on for hemorrhoids. Since then he has been suffering from an increase of cough, expectoration, and fever; at the same time night sweats and dyspnea appeared; could not walk because of emaciation. Appetite poor.

Diagnosis.—Pulmonary tuberculosis in the third stage, and hemorrhoids.

Present Illness.—Constitution robust; nourishment impaired; skin and mucous membrane very anemic; features agonal; larynx somewhat congested; neck long; no swollen lymphatic glands; thorax flat; on the left side breathing impaired; dullness down to the second rib on the frontal side of the right lung; on the same side slight dullness down to the costal margin; all the remainder dull; at the region

where slight dullness is elicited bronchial sounds are heard on auscultation; no respiratory sounds in the region of the dullness; slight dullness down to the fifth intercostal space on the frontal side of the left lung; at the second intercostal space tympanic sounds at some points; on the lateral side dullness over a region as wide as three fingers; bronchial respiratory sounds are heard over these regions; normal precardial dullness; no change in the heart sound. Von Pirquet reaction, +, +, +. The bacilli in the sputum correspond to Gaffky's No. III. Temperature 38.9°C. Pulse 108, weak. Weight 50.1 kilos.

Progress.—The first injection of 16 mg. was given on Jan. 19, 1915, the second (16 mg.) on Feb. 2. Temperature fell to 37.3–37.7°C. on Feb. 9. The third injection (16 mg.) was given on Feb. 16. By this time the general condition improved markedly. The fourth injection (17 mg.) was given on Mar. 2. The temperature rose to 38°C. and the bacilli in the sputum also increased to Gaffky's No. VII. The physical phenomena were intensified until an interval of 3 weeks had elapsed, when the temperature fell to 37.1°C. The fifth (16 mg.), the seventh (15 mg.), and the eighth injections (16 mg.) were given on Apr. 7 and 20, and May 4, respectively. At present the temperature is about 37°C. The physical phenomena are represented in the accompanying charts. Though improvement is obvious, the bacilli in the sputum have not disappeared.

Case 11.—N. T., male, aged 23 years. Father living; mother died of typhoid fever; seven paternal uncles and aunts, three of whom died of an indefinite disease; one is suffering from tuberculosis; all the rest are healthy; no brothers or sisters.

The patient has been weak since birth; contracted whooping cough in his 8th year; ever since has been unwell during the winter; contracted pneumonia once; has empyema of the maxillary sinus; contracted apical tuberculosis 3 years ago, which subsided after clinical treatment, but is not yet completely cured; rarely has pain in the thorax; appetite good; stools once daily.

Diagnosis.—Pulmonary tuberculosis in the first stage.

Present Illness.—Constitution and nourishment good; skin and mucous membrane not anemic; larynx slightly congested; neck long; no swelling of the jugular lymphatic glands; thorax symmetrical; breathing symmetrical; right apex slightly resistant at the supraclavicular fossa; precardial dullness reaches as far as the breasts; on auscultation no change is perceptible; bowels normal. Von Pirquet reaction, +, +, +. Weight 57.2 kilos. Temperature 37°C. Pulse 74, regular. No tubercle bacilli in the sputum.

Progress.—The first injection of 16 mg. was given on Dec. 11, 1914. Many râles appeared along the line bordering the right lung and the liver, as is shown in the chart; the apex showed slight dullness with a few râles; many râles were heard about the region corresponding to the heart; on the lateral side of the right lung slight dullness was produced over a region as wide as three fingers, where some râles were also heard. Tubercle bacilli in the sputum correspond to Gaffky's No. II. These phenomena completely disappeared in 7 days. The second injection (16 mg.), the third (16 mg.), and the fourth (16 mg.) were given

on Dec. 26, Jan. 8, and 22, respectively. No reaction was produced, and the results of the fifth injection (17 mg.) brought about some reaction. Since then four injections were made, but no reaction was produced. All examinations of the sputum have been negative. Von Pirquet negative. Treatment suspended.

SUMMARY.

A general review of the cases will, I think, indicate that the preparation[2] greatly improves or apparently cures pulmonary and surgical tuberculosis in the first and second stages, and that it seems also to produce beneficial effects upon the disease in the third stage. The duration of these beneficial effects is still to be established by more numerous trials and many years of observation.

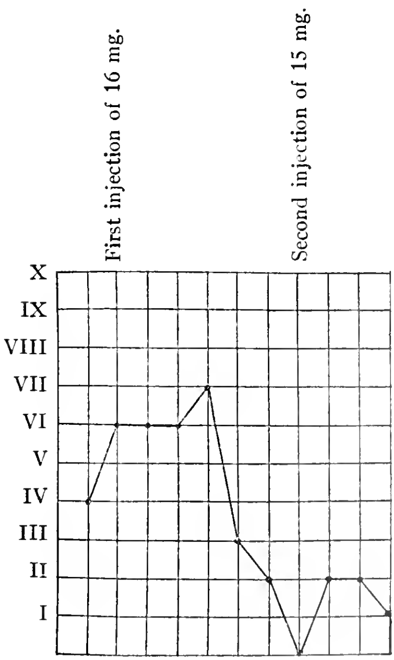
The preparation must be given intravenously, and the doses must be increased or decreased according to the age and constitution of each patient (page 156). Moreover, it should be borne in mind that the pathological phenomena and the constitution of each patient have much to do with the determination of the dose. The manner of action of the preparation is not yet entirely clear. But if it acts primarily upon the tissues which bear the tubercular lesions and then indirectly against the germ, as I assume at present, the activity which the tissues exert will have much to do with the efficacy of the preparation. If this hypothesis is correct, the minimum doses (10 to 12 mg.) will be best suited to a patient who is greatly emaciated, and should be gradually increased as the reactions, pathological processes, nutrition, etc., indicate. In any case, the dose of the preparation must be determined by the condition and constitution of the patient. In animal experiments I have been fortunate enough to obtain results which no other preparation has given. The clinical application and the establishment of its full efficacy in human cases must be left to the physician.

I wish to express my indebtedness to Drs. Kitasato, Shiga, and Kusama, and also to Dr. Kanai and other members of the Kitasato Institute.

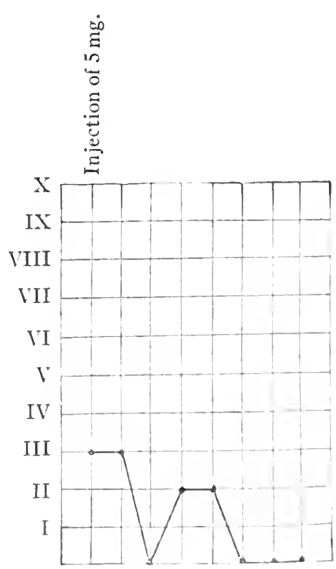
[2] The preparation has since been named "cyanocuprol."

The Roman numerals in Text-figs. 1 to 6 refer to the numbers in Gaffky's table:

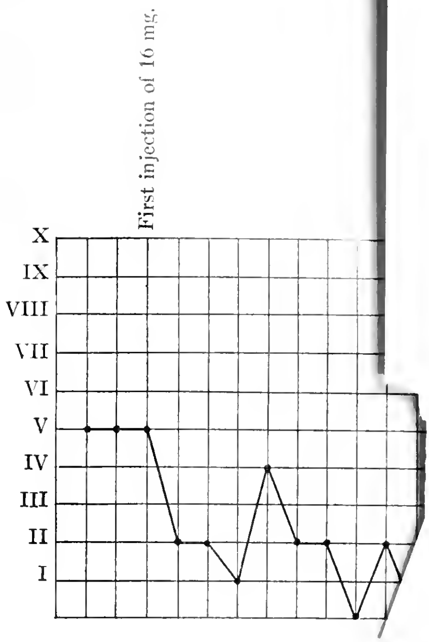
I.	Only 1 to 4	bacilli in whole preparation.						
II.	Only 1	on	an	average	in	many fields.		
III.	Only 1	"	"	"	"	each field.		
IV.	2 to 3	"	"	"	"	"	"	
V.	4 to 6	"	"	"	"	"	"	
VI.	7 to 12	"	"	"	"	"	"	
VII.	13 to 25	"	"	"	"	"	"	
VIII.	About 50	"	"	"	"	"	"	
IX.	About 100	"	"	"	"	"	"	
X.	Over 100 in each field.							



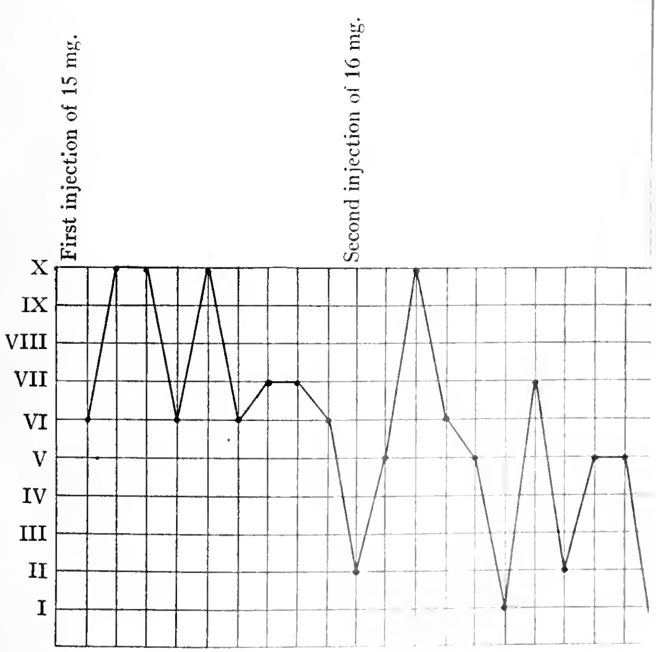
TEXT-FIG. 1. Case 2, male. The sputum is free from bacilli at present.



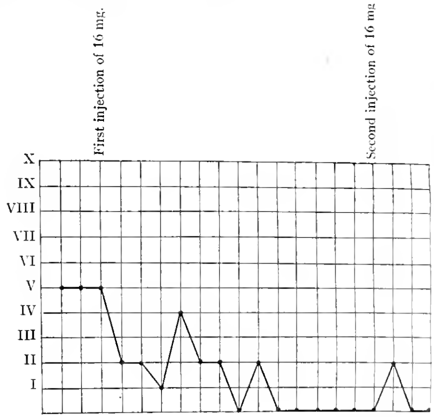
TEXT-FIG. 2. Case 3, male. The patient did not report again and therefore received no further treatment.



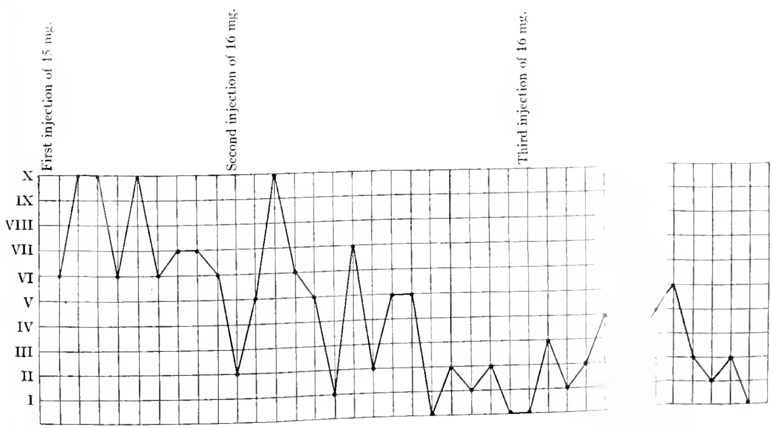
TEXT-FIG. 3. Case 5, male. The sputu
scope; negative results were obtained each t



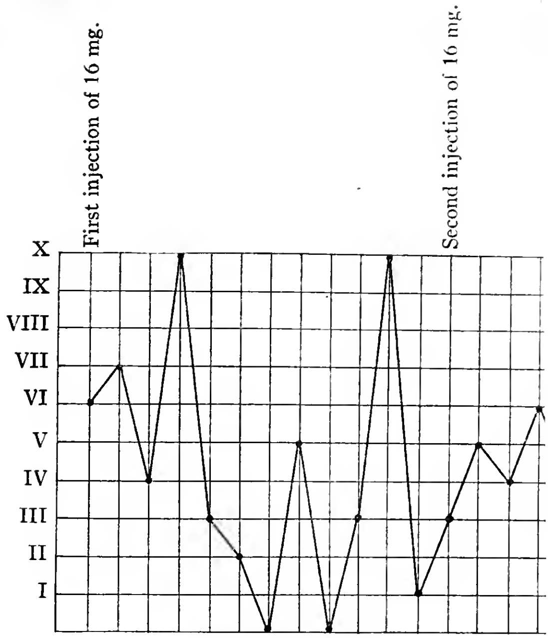
TEXT-FIG. 4. Case 8, male. Every microscopic exa



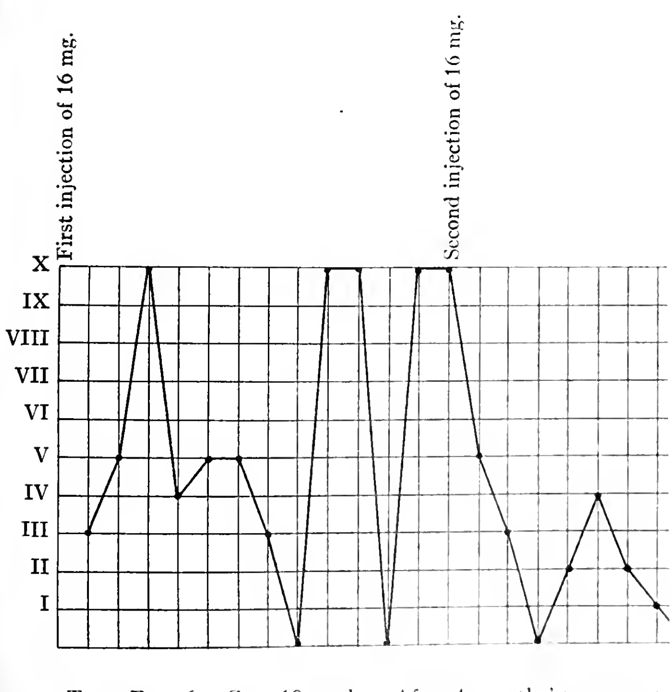
TEXT-FIG. 3. Case 5, male. The sputum was examined under the microscope; negative results were obtained each time.



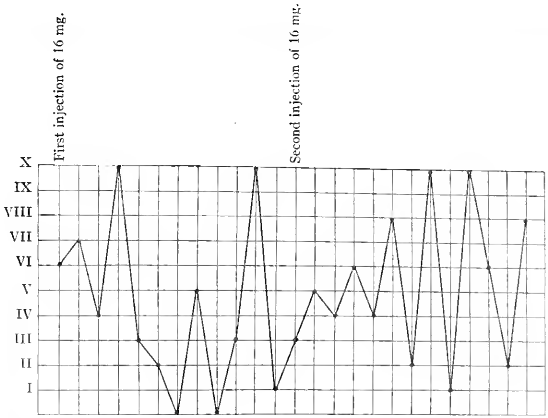
TEXT-FIG. 4. Case 8, male. Every microscopic examination has been negative for more than 4 months.



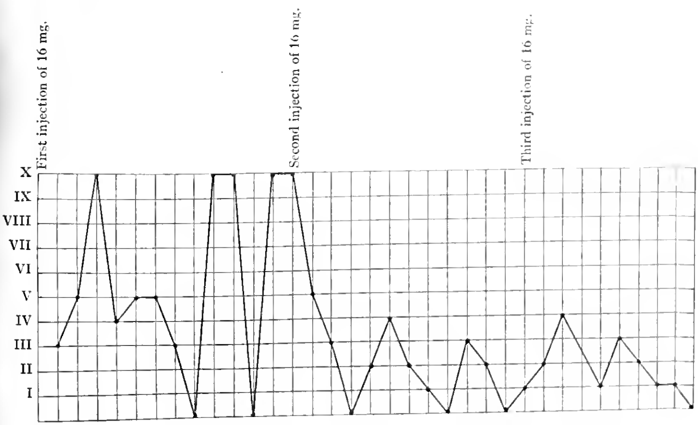
TEXT-FIG. 5. Case 9, female. The symptoms b
of bacilli in the sputum increased.



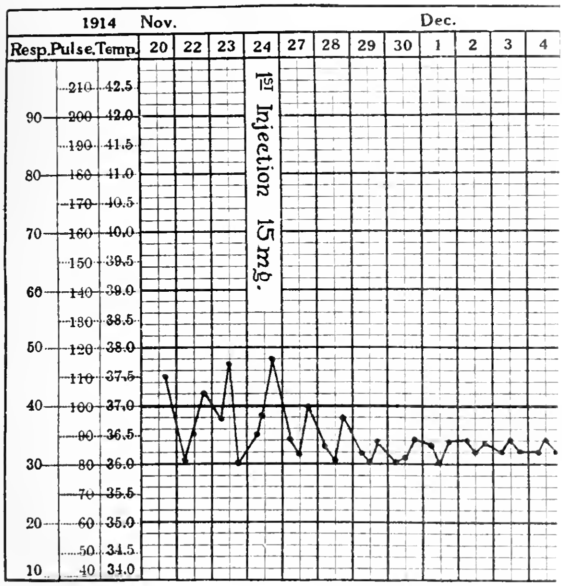
TEXT-FIG. 6. Case 10, male. After 4 months' treatment



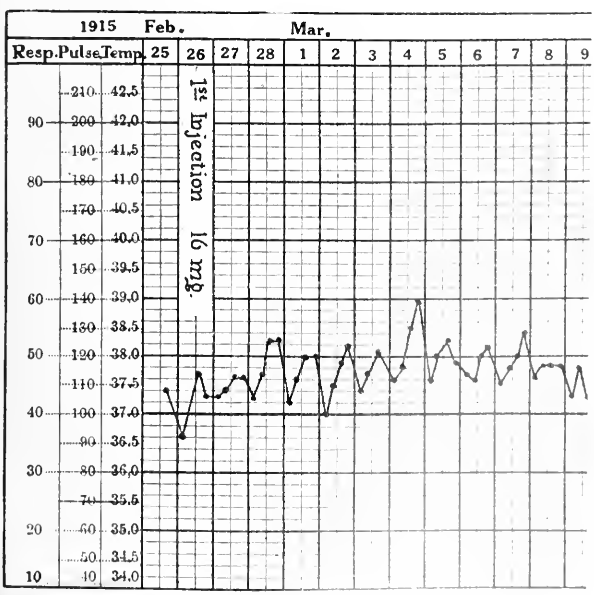
TEXT-FIG. 5. Case 9, female. The symptoms became worse and the number of bacilli in the sputum increased.



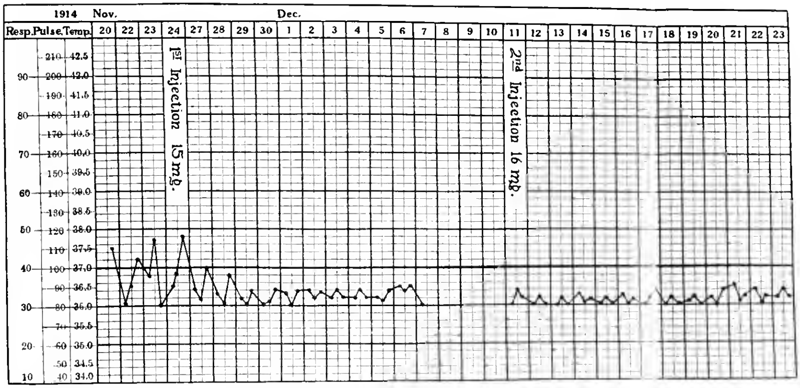
TEXT-FIG. 6. Case 10, male. After 4 months' treatment the sputum was free from bacilli.



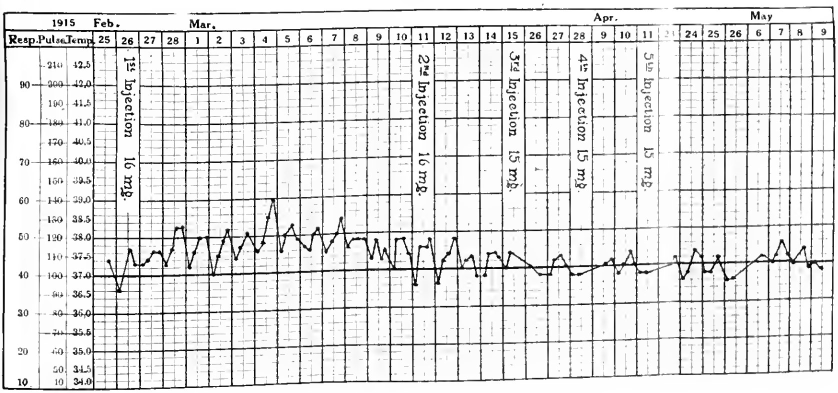
TEXT-FIG. 7. Tem



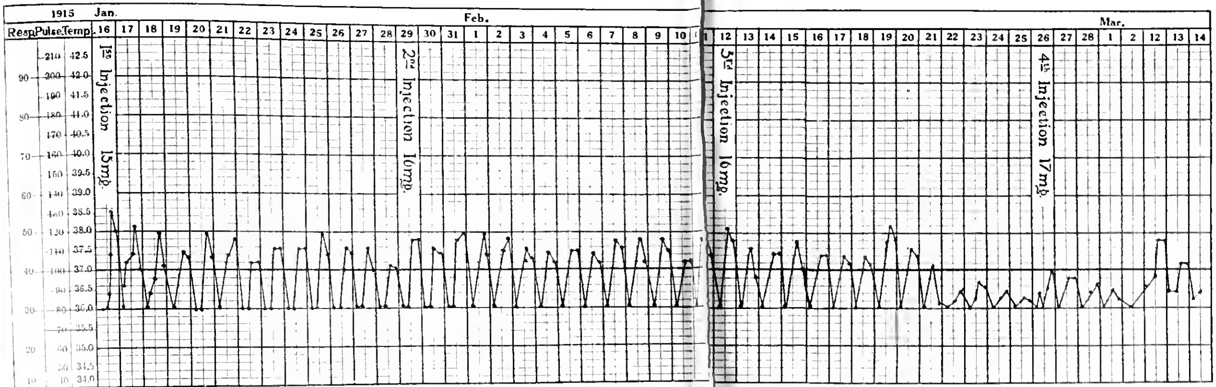
TEXT-FIG. 8. Ten



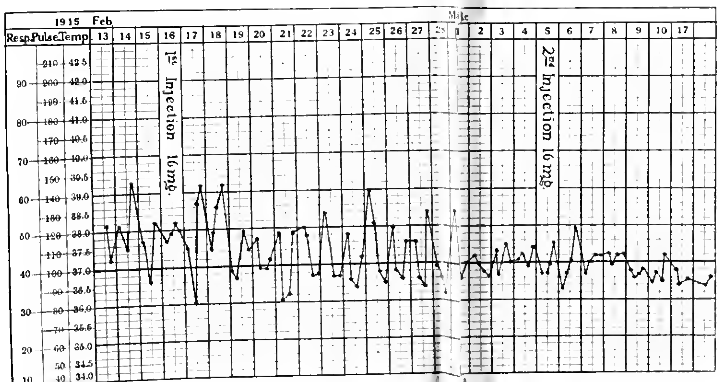
Text-Fig. 7. Temperature curve. Case 1, female.



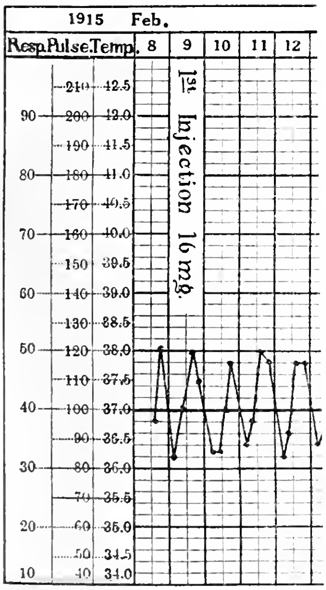
Text-Fig. 8. Temperature curve. Case 3, male.

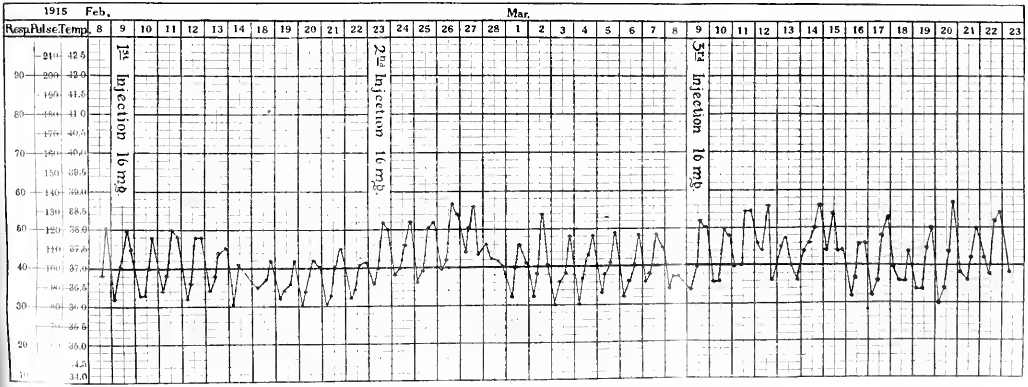


TEXT-FIG. 9. Temperature curve. Case 8, male.

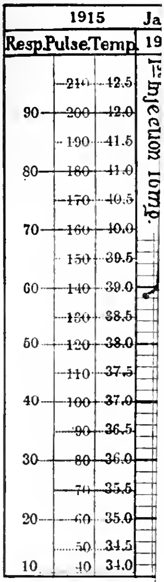


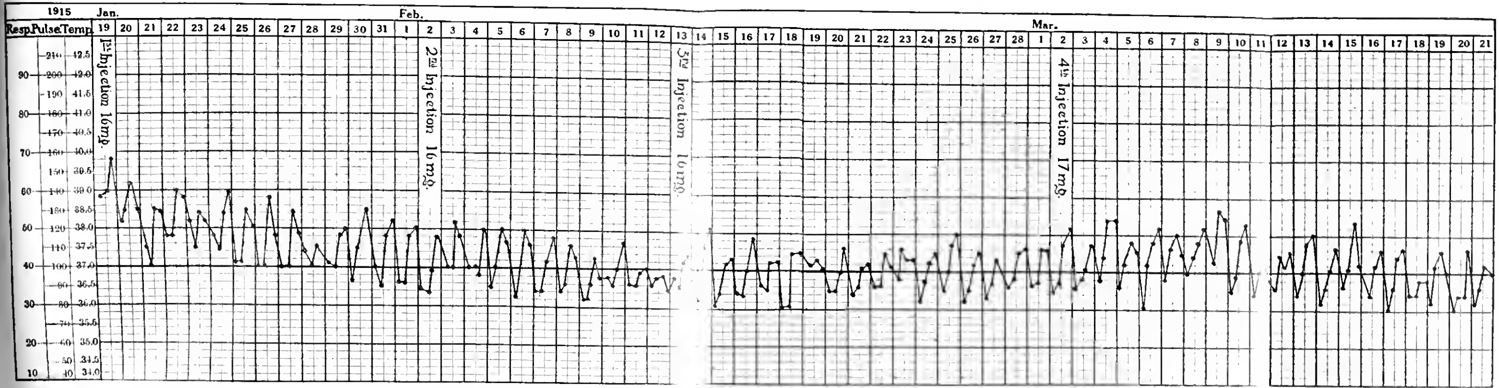
TEXT-FIG. 10. Temperature curve. Case 4, female.


1915 Feb.
Resp. Pulse. Temp.
8
9
10
11
12
1st Injection 16 mg.
90
80
70
60
50
40
30
20
10
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
42.5
42.0
41.5
41.0
40.5
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
34.5
34.0

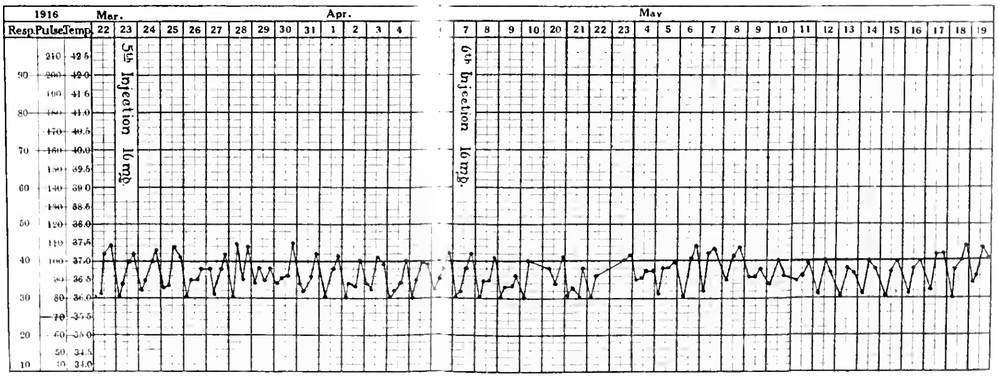


TEXT-FIG. 11. Temperature curve. Case 9, female.


1915
Ja
Resp. Pulse. Temp.
19
1st Injection Temp.



TEXT FIG. 1 emperature curve. Case 10, male.



TEXT-FIG. 13. Temperature curve. Case 10 continued.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

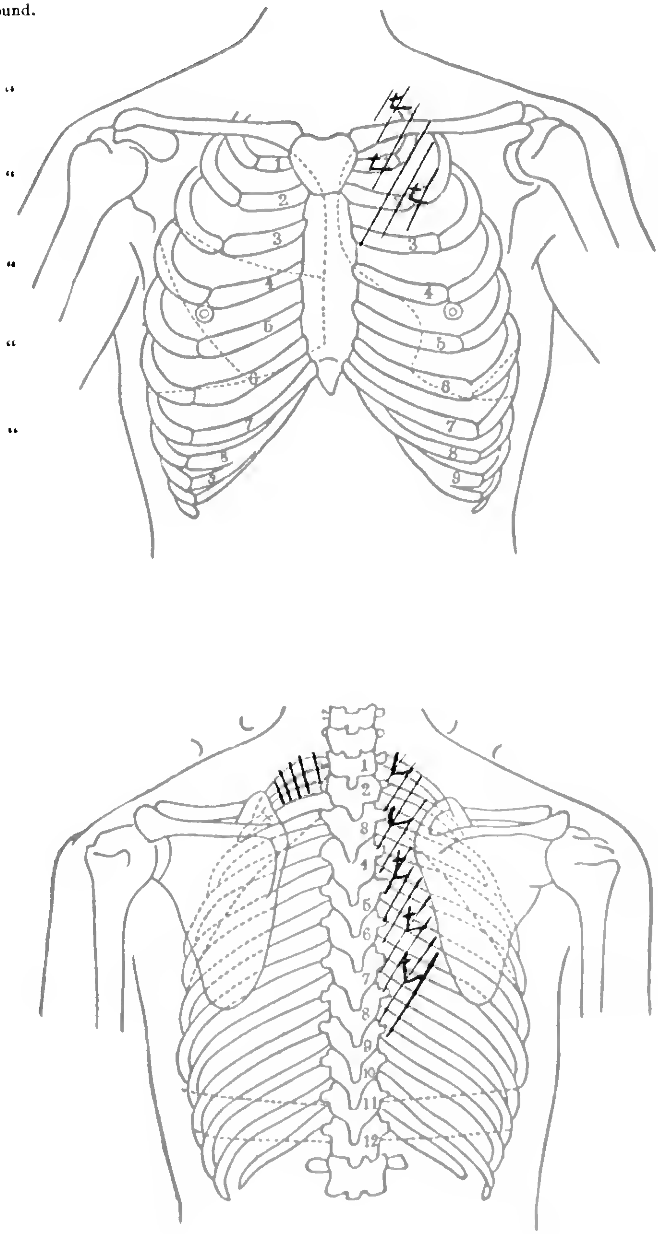
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 14. Case 6, male. Nov. 11, 1914.

Bronchial respiration sound

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

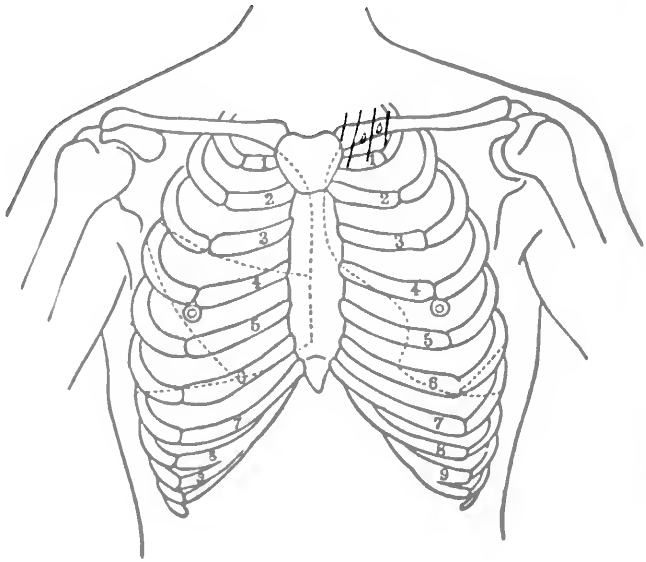
S Sonorous "

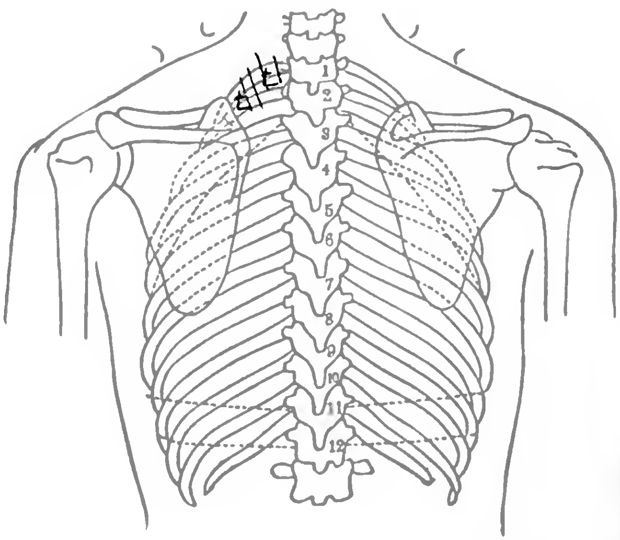
Short sound.

Dull "

ty Metallic "

Heart "





TEXT-FIG. 15. Case 6, male. Nov. 26, 1914.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

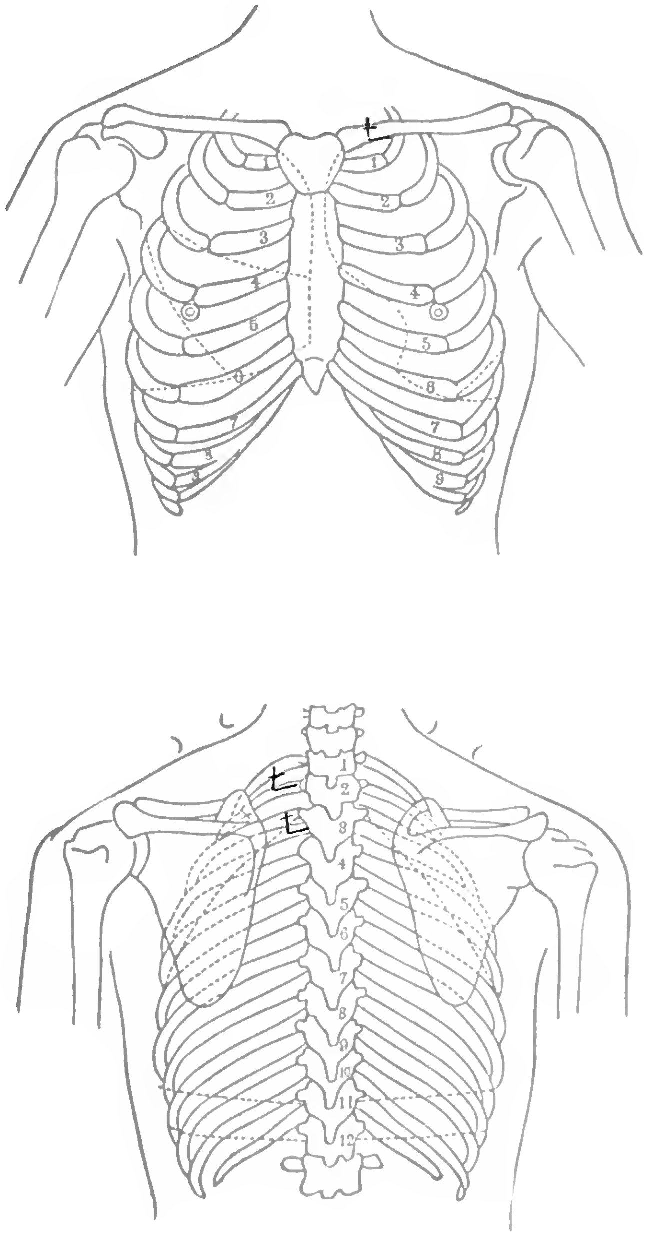
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 16. Case 6, male. Dec. 8, 1914.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

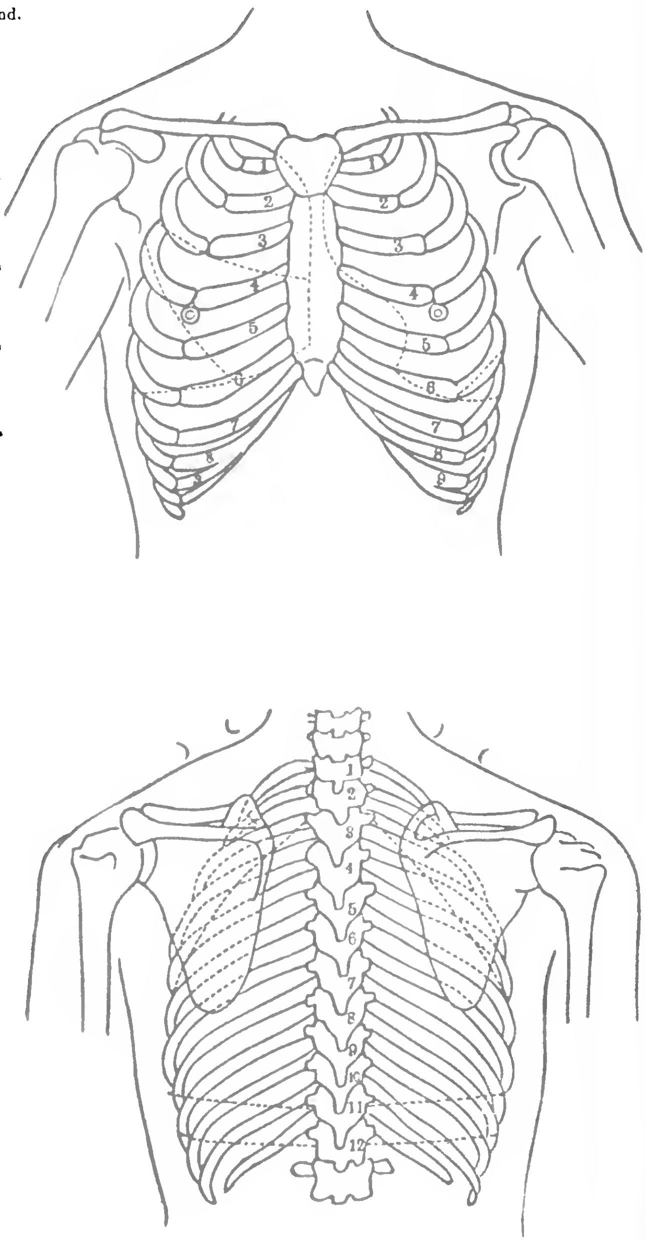
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



Text-Fig. 17. Case 6, male. Jan. 12, 1915, no symptoms.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

Sibilant "

Crackling "

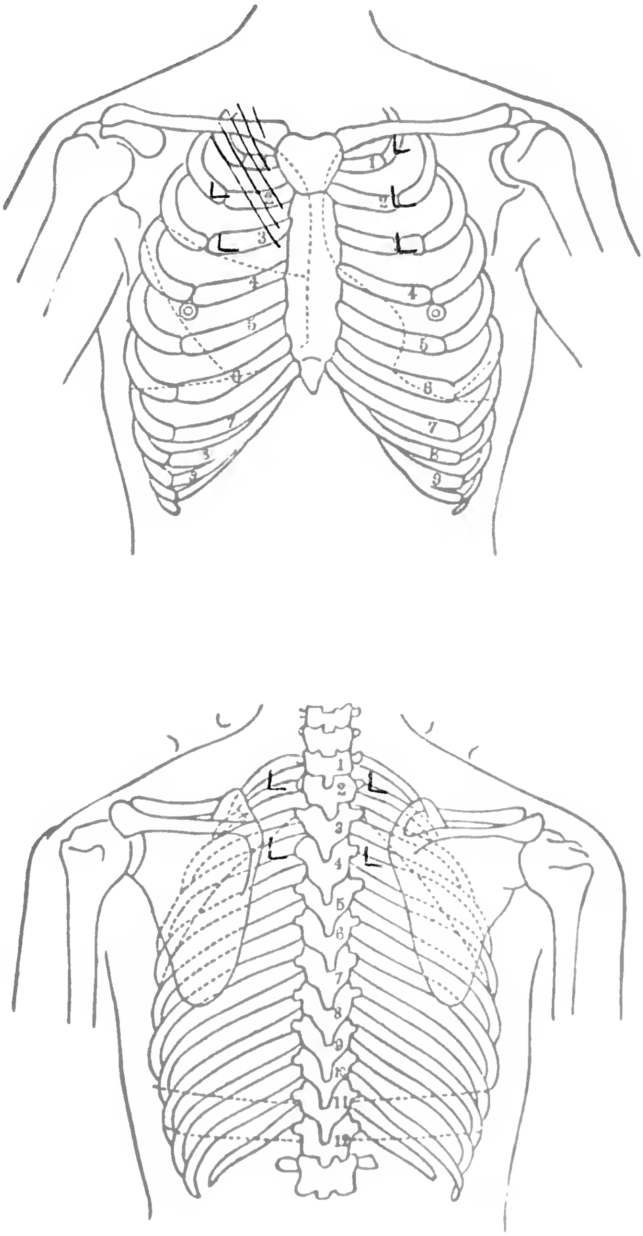
Sonorous "

Short sound.

Dull "

Metallic "

Heart "



TEXT-FIG. 18. Case 7, male. Feb. 16, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

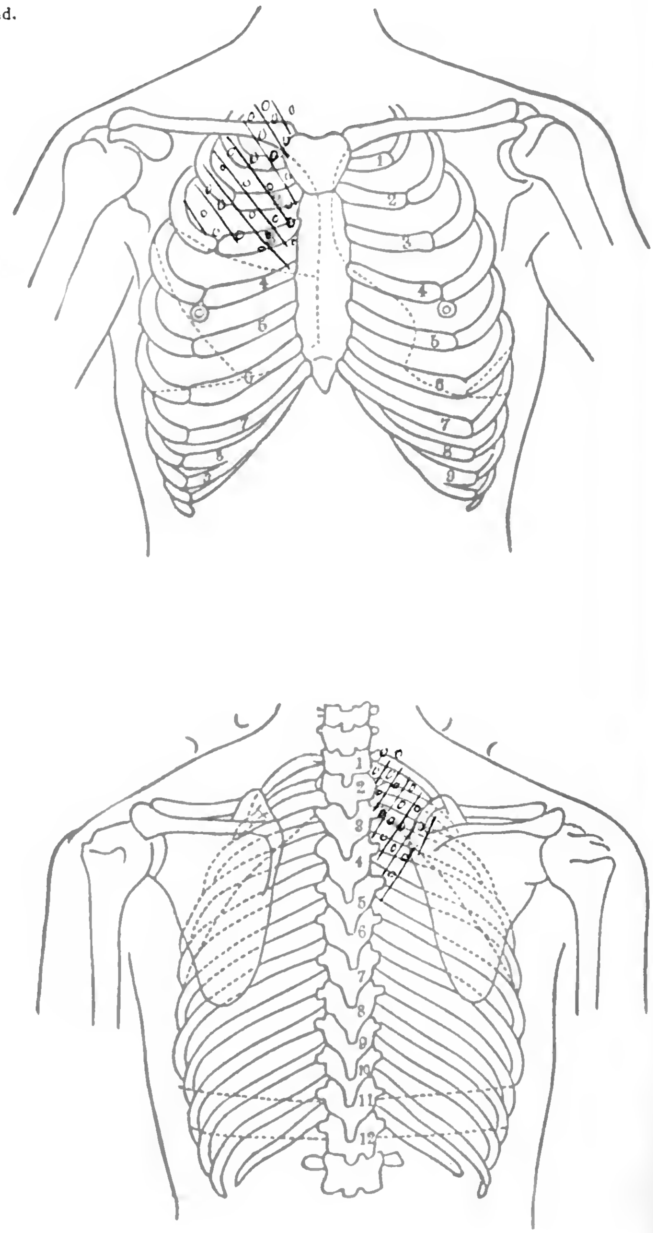
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



Text-Fig. 19. Case 7, male. Feb. 20, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

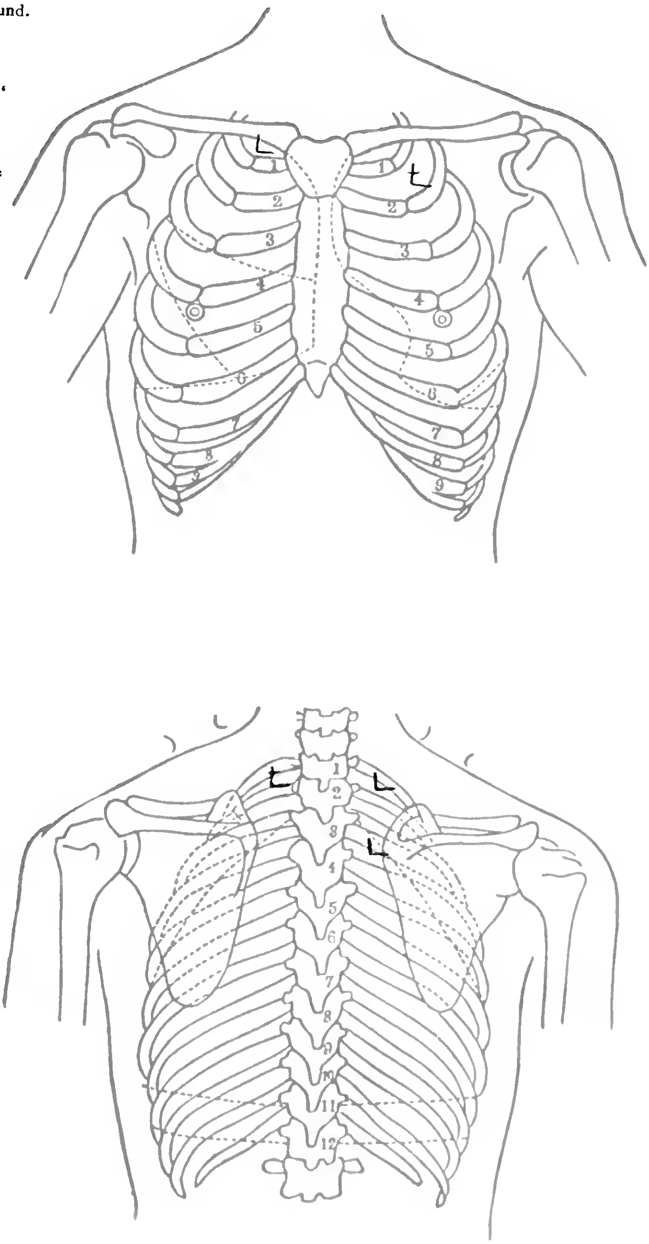
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 20. Case 7, male. Feb. 26, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

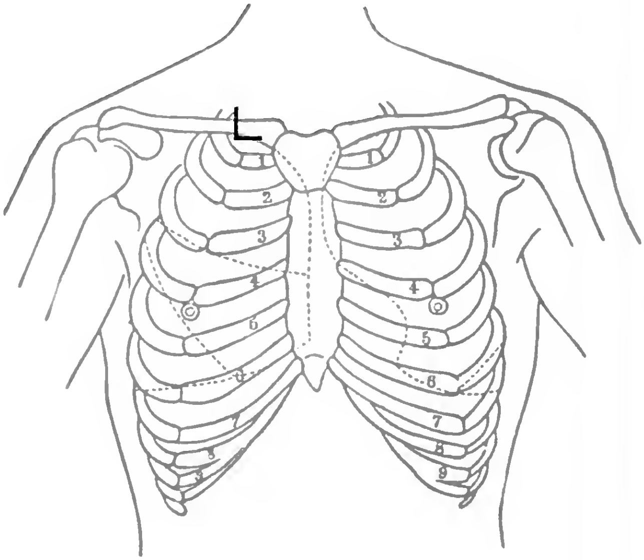
S Sonorous "

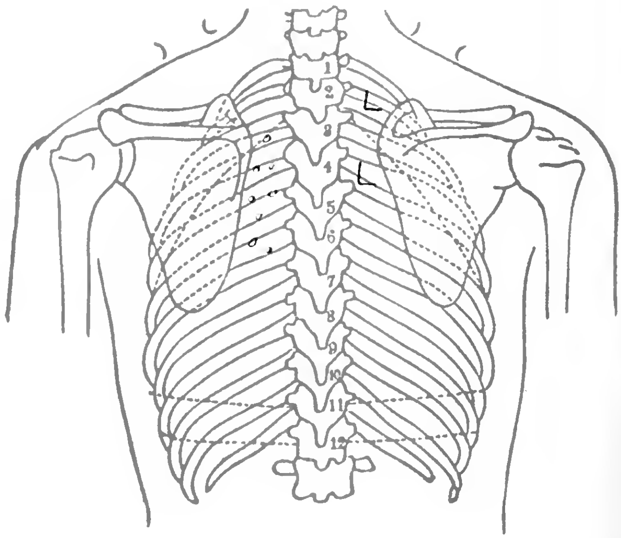
Short sound.

Dull "

ty Metallic "

Heart "





TEXT-FIG. 21. Case 7, male. May 7, 1915. Note the appearance of râles in the intrascapular region, after an interval of 3 months during which time he did not receive the injection.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

Sibilant "

Crackling "

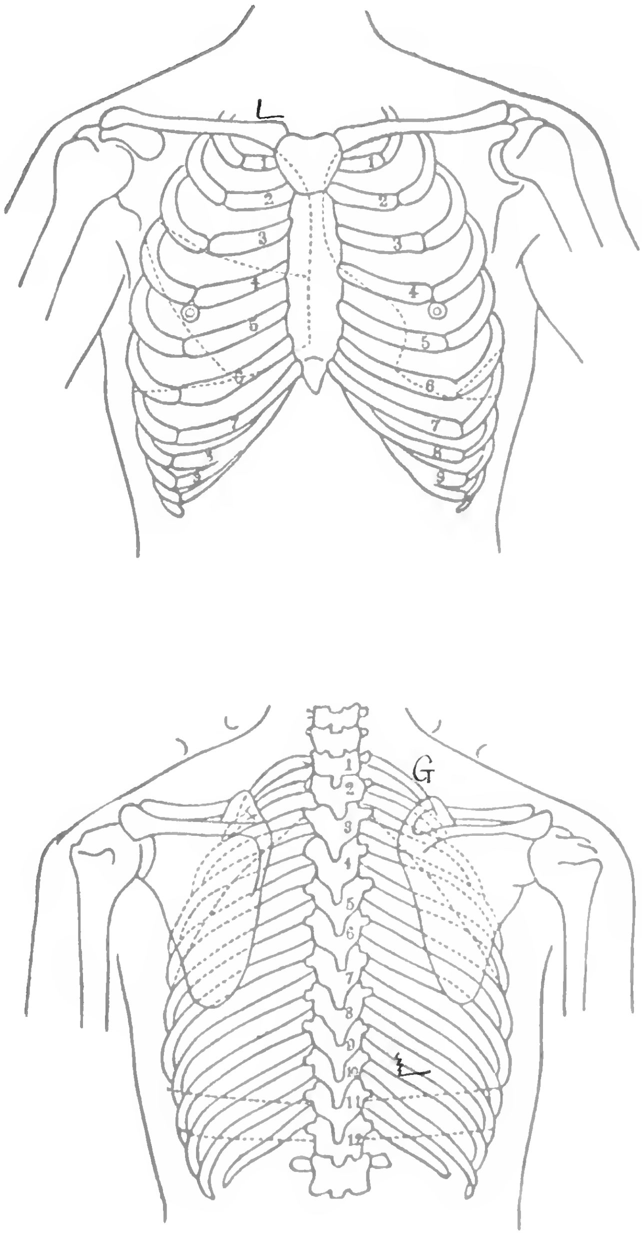
Sonorous "

Short sound.

Dull "

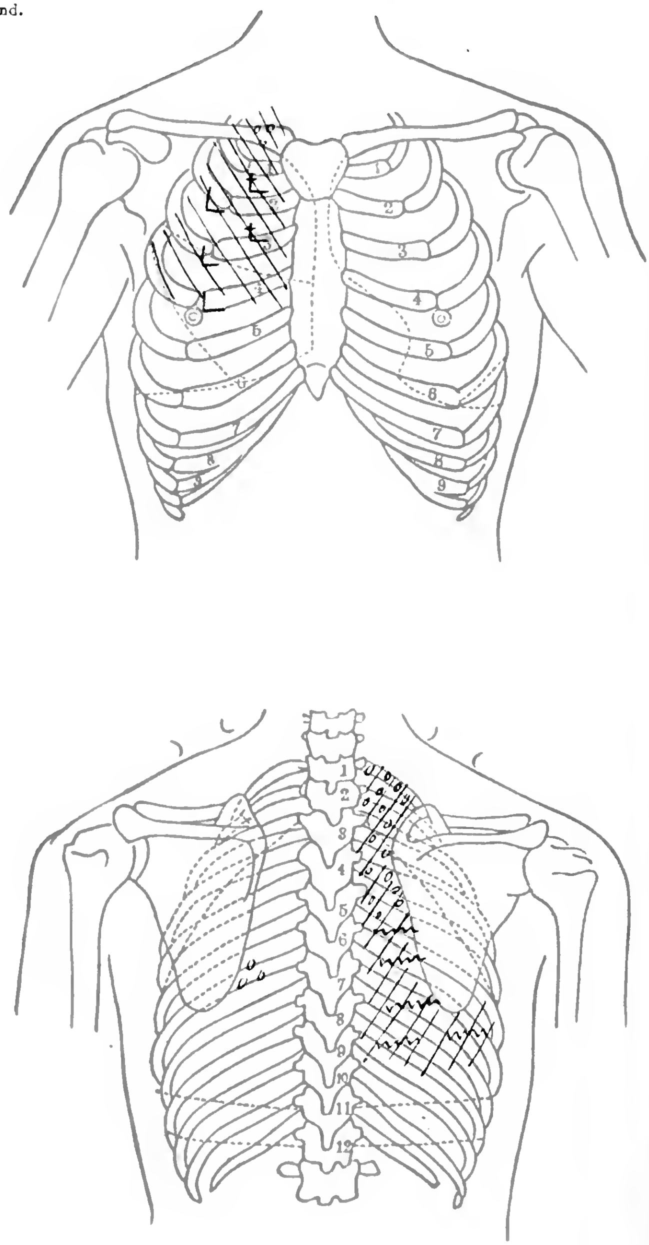
Metallic "

Heart "



TEXT-FIG. 22. Case 7, male. May 20, 1915. The râles disappeared after one injection of 15 mg. and the injection was discontinued.

Bronchial respiration sound.
Weak " "
Loss of " "
Rough " "
Interrupted " "
Long " "
Coarse râles.
Fine "
Consonant râles.
Vesicular "
Friction "
Sibilant "
Crackling "
Sonorous "
Short sound.
Dull "
Metallic "
Heart "



TEXT-FIG. 23. Case 9, female. Feb. 8, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

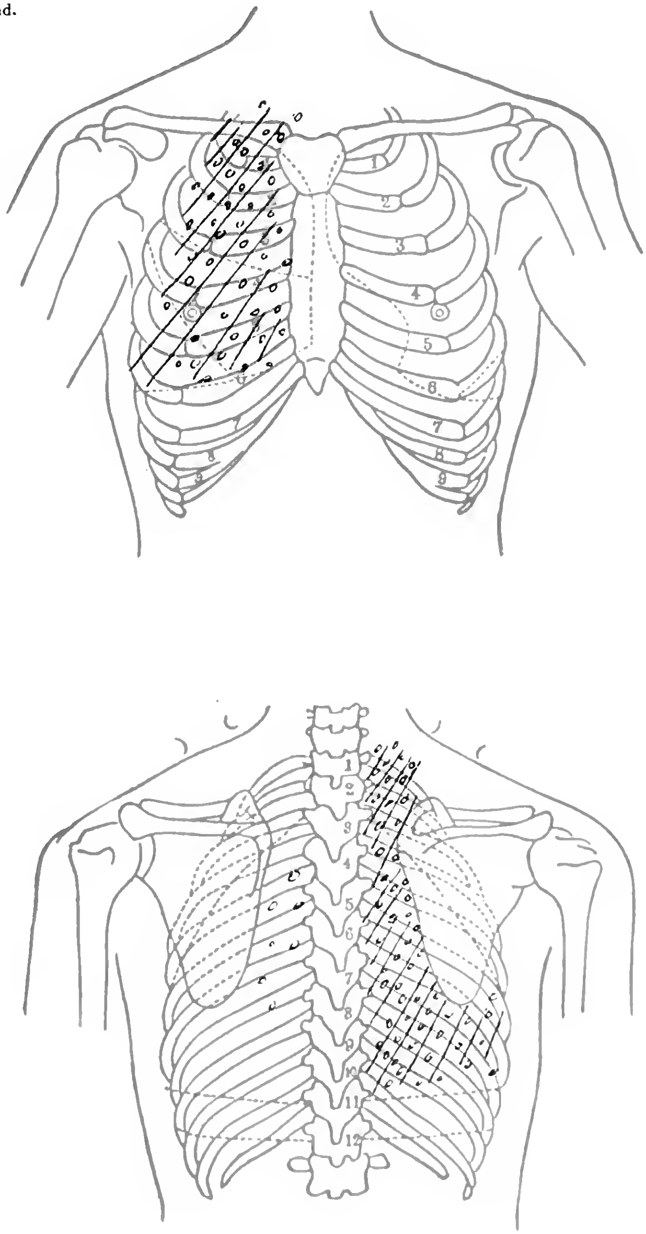
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT FIG 24. Case 9, female. Apr. 19, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles

Fine "

Consonant râles.

Vesicular "

Friction "

Sibilant "

Crackling "

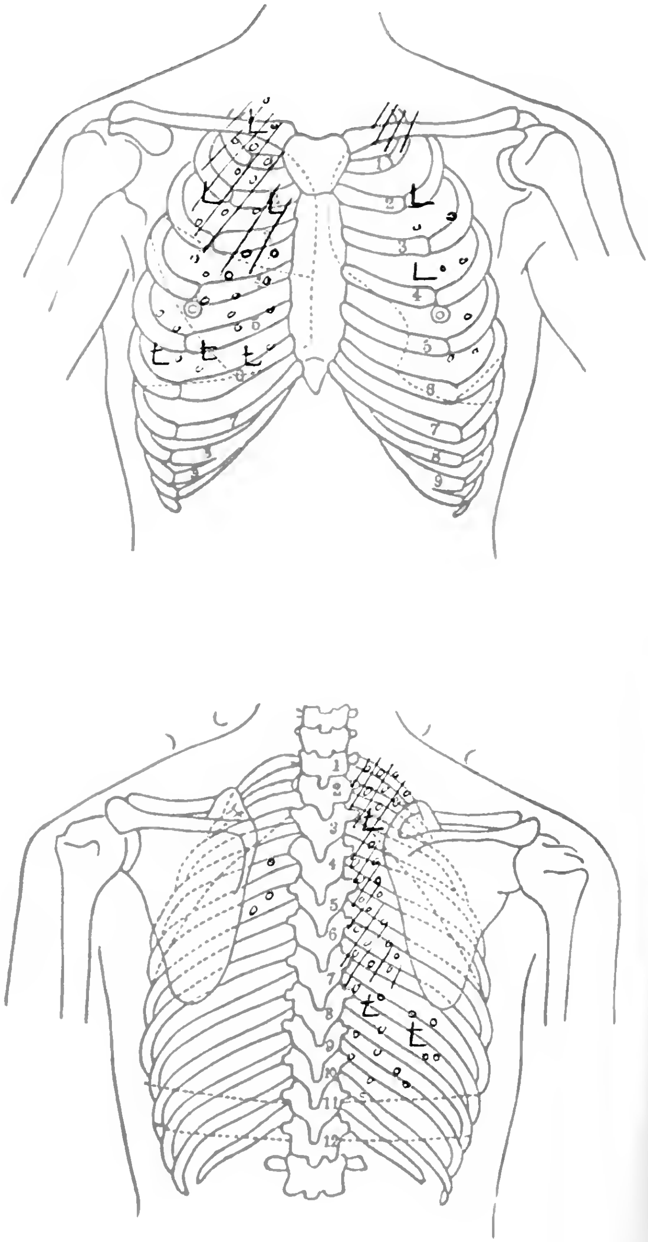
Sonorous "

Short sound.

Dull "

Metallic "

Heart "



TEXT-FIG. 25. Case 9, female. Apr. 27, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

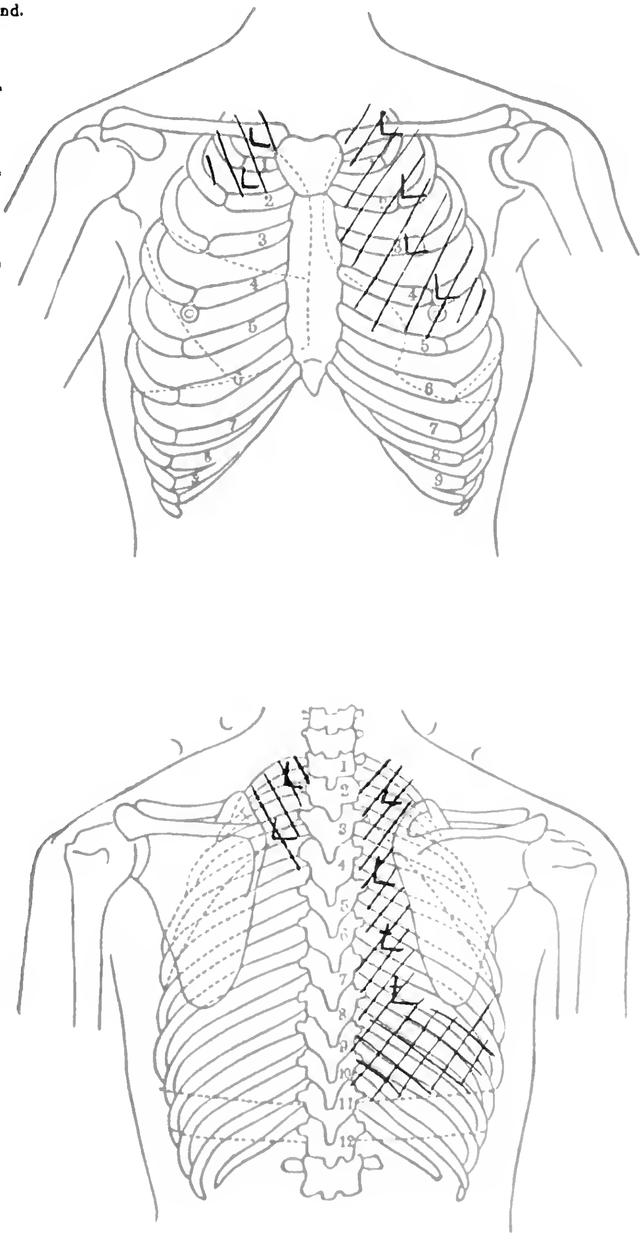
S Sonorous "

Short sound.

Dull "

ty Metallic

Heart



Text-Fig. 26. Case 10, male. Jan. 19, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

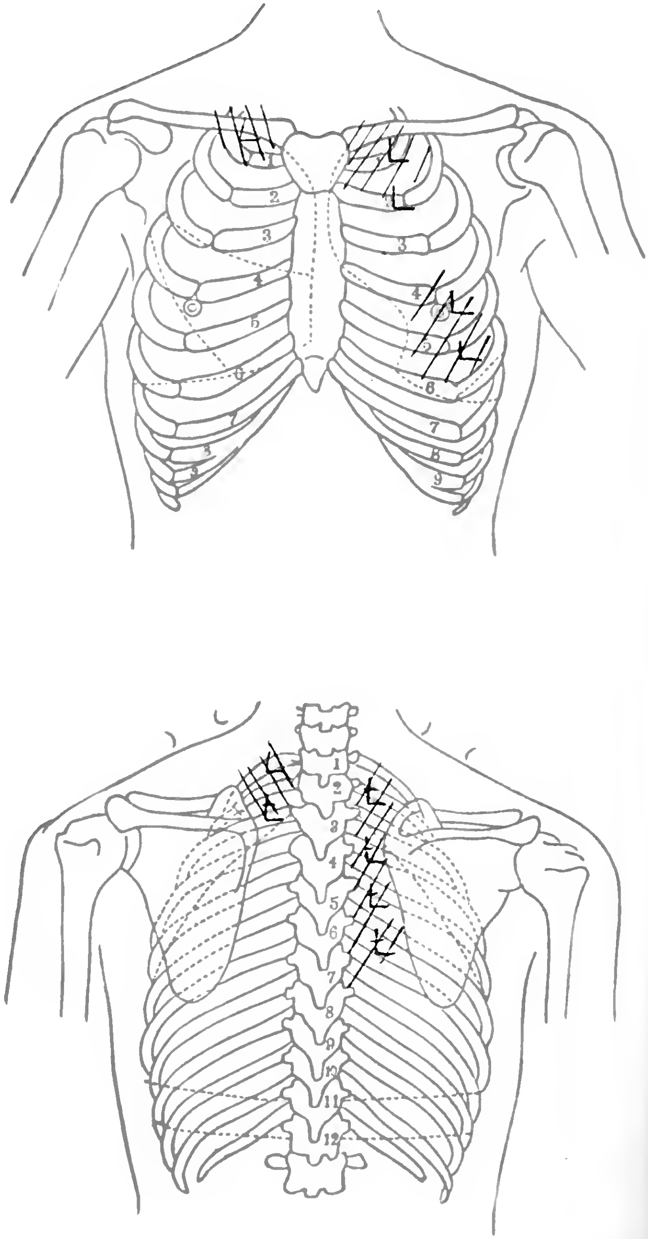
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 27. Case 10, male. Feb. 13, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

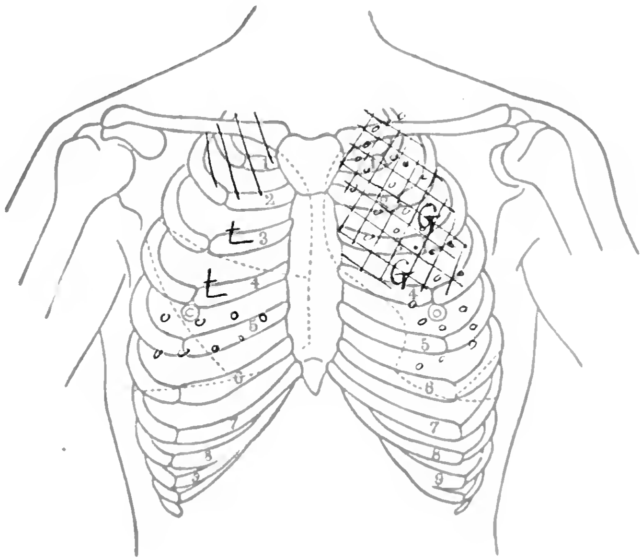
S Sonorous "

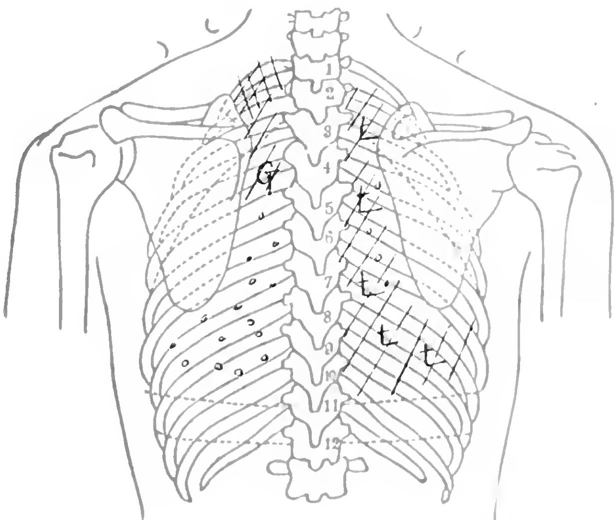
Short sound.

Dull "

ty Metallic "

Heart "





TEXT-FIG. 28. Case 10, male. Apr. 20, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

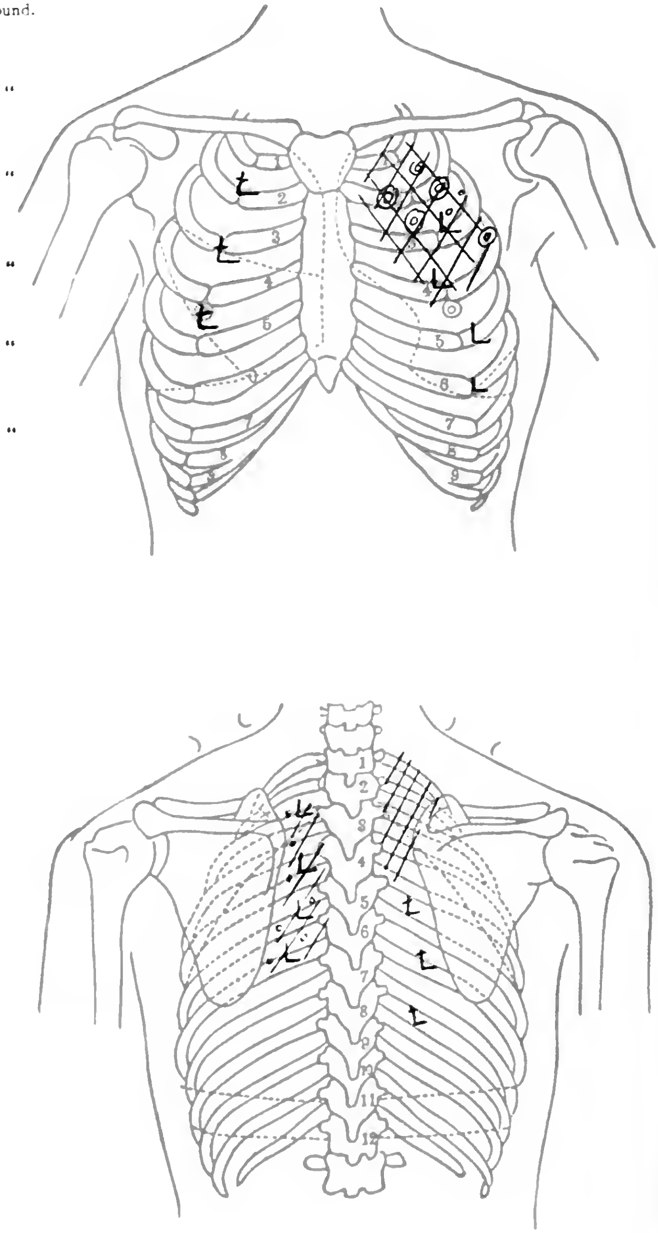
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 29. Case 10, male. May 17, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

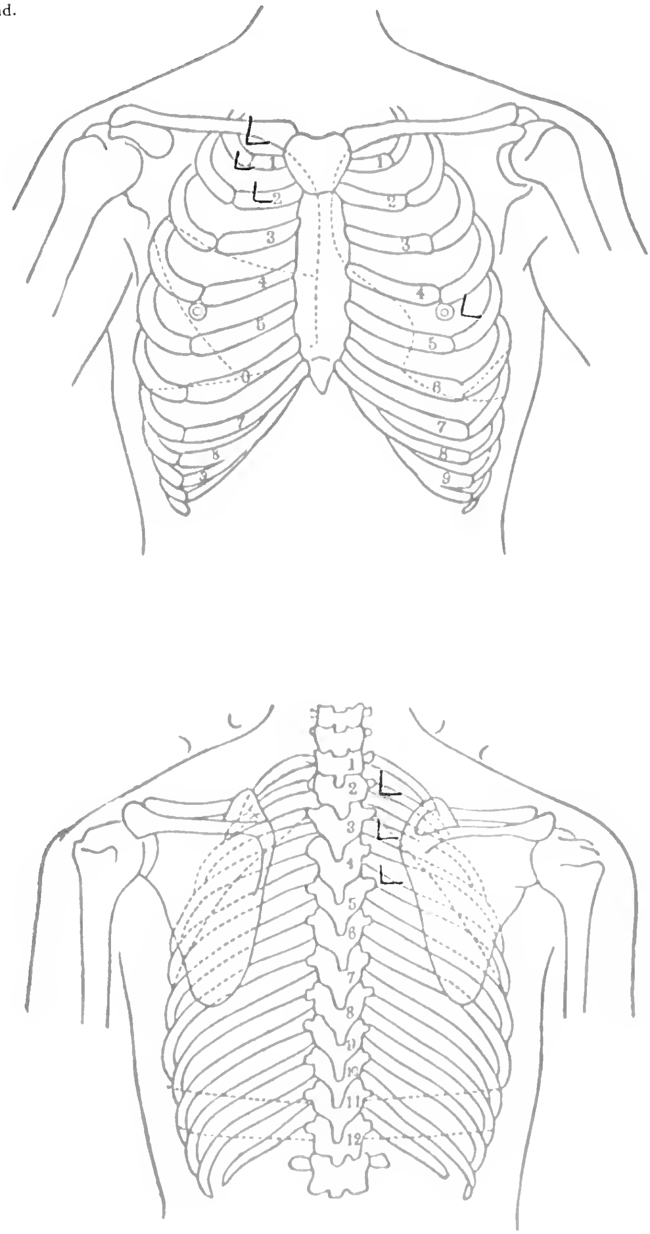
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 30. Case 11, male. Dec. 3, 1915.

Bronchial respiration sound.

Weak " "

Loss of " "

Rough " "

Interrupted " "

Long " "

Coarse râles.

Fine "

Consonant râles.

Vesicular "

Friction "

P Sibilant "

G Crackling "

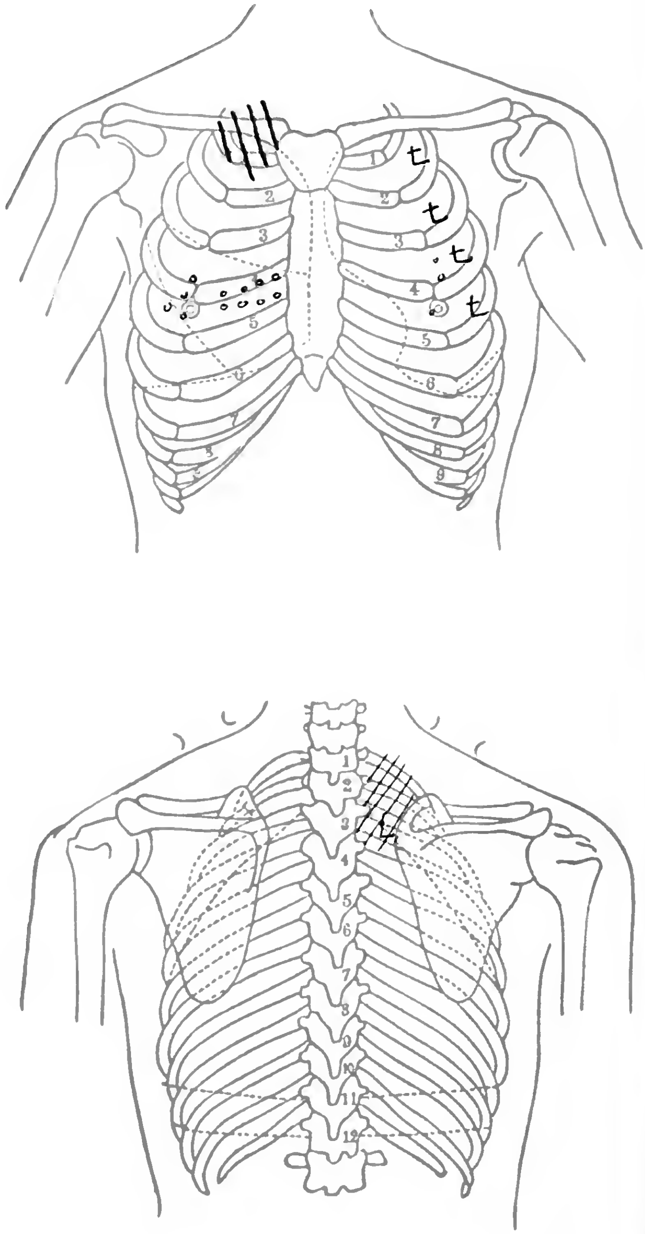
S Sonorous "

Short sound.

Dull "

ty Metallic "

Heart "



TEXT-FIG. 31. Case 11, male. Dec. 11, 1915.

THE TREATMENT OF TUBERCULOSIS WITH CYANOCUPROL.

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The present paper deals with the clinical treatment of tuberculous patients with cyanocuprol. I believe it desirable to report the work as far as it has progressed, although the cases are still too few to permit conclusions regarding the efficacy of the preparation. A later communication covering this point may be expected.

General Considerations.

Koga[1] reported that the intravenous injection of cyanocuprol brings about a degree of reaction in tuberculous cases, which is proportional to the amount given in each instance. However, the relation between the reaction and the efficacy has not yet been definitely established. Too great a reaction produced severe effects on the patient and smaller doses have therefore been given. The reactions have thus been eliminated, but the results seem incidentally to have become inferior to those obtained by Koga with the larger doses. The tubercle bacilli in the sputum, also, did not show such a marked decrease as under the influence of larger doses.

Although cyanocuprol produces favorable results in the condition taken as a whole when administered in adequate doses, yet indifferent cases sometimes occur in which its efficacy is doubtful. This indifferent result may depend upon insufficient dosage. Moreover, the constitution of the patient and the nature of the pathological processes play an important part in determining the effects.

Indications and Contraindications.—Cases suitable for the application of cyanocuprol are far more numerous than for the tuberculin

[1] Koga, G., *J. Exp. Med.*, 1916, xxiv, 149.

treatment, since it may be administered in almost all instances of tuberculosis. Even in the final stages the preparation may be given without danger, although with little hope of improvement. Patients suffering from progressive tuberculosis in the third stage sometimes gave an unexpectedly good result, and high fever abated after an intravenous administration. Pulmonary hemorrhages sometimes occurred during the reaction period, but they were traced invariably to carelessness on the part of the patient. The data at hand suffice to state that the preparation does not influence injuriously pulmonary hemorrhages but rather increases the coagulability of the blood so that the hemorrhage may be arrested. Cases with fever in consequence of cold may be given the drug without inconvenience. However, it may be advisable to wait until recovery from the cold takes place before giving the drug. The drug may be given to tuberculous patients with chronic diseases of the heart and kidney, and glycosuria and beri-beri, but it must not be given to patients having irregular or accelerated pulse (*e.g.*, over 120) or weak tonus of the pulse. The preparation may be given during menstruation and pregnancy provided care be exercised to avoid any unusual reaction.

Dosage.—There exists an intimate relation between the proper dose and the condition of the patient, for upon the dose seems to depend largely whether or not improvement follows. We are now studying this point with the utmost care. It seems to be much more difficult to ascertain the proper dose of cyanocuprol for each individual than of tuberculin. For instance, in some cases a reaction set in in consequence of an increase of the dose by 0.25 cc., in others a rise of temperature occurred even when the same dose was given each time. The dose of tuberculin may be settled comparatively easily by the degree of von Pirquet reaction or the reaction produced by the intracutaneous injection of tuberculin. But with cyanocuprol we have as yet no preparatory test by which the dose can be determined. We are at present determining, according to our past experiences, the amount for each individual case by the following standard doses, which have been chosen with reference to the site and severity of the pathological processes.

TABLE I.

Type of disease.			Dose. cc.
Pulmonary	tuberculosis,	1st stage	7.0–7.5
"	"	2nd "	6.0–7.0
"	"	3rd "	4.0–6.0
Laryngeal	"		4.0–6.0
Intestinal	"		4.0–6.0
Pleural	"		4.0–6.0
Peritoneal	"		4.0–6.0
Kidney	"		3.0–5.0
Bone	"		7.0–8.5
Joint	"		7.0–8.5
Cutaneous	"		7.0–8.5
Glandular	"		7.0–8.5

Table I gives the standard dose for adult men, and must be altered according to the general condition of the patient. For instance, patients having high fever, showing a tendency to pulmonary hemorrhage, suffering from neurasthenia, malnutrition, extreme anemia, or showing marked progress of the pathological processes, heart disease, or chronic diseases other than tuberculosis, or presenting a wide tuberculous lesion, etc., should receive smaller doses. The body weight would naturally seem to influence the dose, but my experience has led me to the conclusion that there is no need to vary the dose in proportion to the body weight. However, it must be decreased according to the patient's age; *e.g.*, a boy 10 years old should receive half the dose given to an adult man. The dose for the second injection should be made according to the degree of the reactions produced by the first, as well as by the general condition of the patient at the time of the injection. By a proper dose is meant the amount of the preparation which produces no or only a slight reaction, which disappears 2 or 3 days after the injection, and shows some improvement within 2 weeks. If these results are achieved, the dose should not be altered but should be continued for several injections until a change is deemed desirable. If too great a reaction, *i.e.*, fever exceeding 1°C., is produced, or if a local reaction lasting more than 3 days should occur, the dose must be decreased. If, on the contrary, neither reaction nor improvement is observed, the dose is increased by 0.25 cc. at the next injection. In

severe cases or cases suffering from progressive lesions, the increase must be made with special care.

Interval between Injections.—Great care should be exercised to allow a sufficient interval between the injections, as the interval as well as the dose seems to have much to do in bringing about improvement. The doses shown in Table I are the amounts that should be given once every other week. The injection should never be given with a shorter interval than a fortnight, as otherwise an unfavorable turn in the general symptoms may follow. Takano[2] reports that he gave lepers large doses weekly or sometimes twice a week, and observed no ill effects. But with tuberculosis such an attempt should not be made. Our experience has convinced us that Koga's view, based on animal experiments, of allowing an interval of 2 weeks between injections is correct. Prolongation of the interval may be made without harmful effects. If, therefore, the reaction produced by the injection should last more than 1 week, the succeeding injection must be postponed.

Reactions and Effects.—Koga has observed that the administration of cyanocuprol may be accompanied by striking reactions. But by diminishing the dose no reaction will be produced, and even if it should appear, no serious symptoms will develop. As a rule, the fever does not rise above 1°C., and the reactions disappear in 2 or 3 days, rarely lasting more than 1 week. The fever is sometimes preceded by a slight chill. Fever does not necessarily accompany the pain in the joints. The general reactions are headache, dullness in the limbs, lack of appetite, etc., and are so slight that no special treatment is called for. A fleeting inflammation appears in the lesion, as pointed out by Koga, but by the improved method of treatment, regional inflammation is avoided. The pulmonary dullness is rarely increased, and if increased it is slight. Râles are often heard, but they disappear in 2 or 3 days with no other treatment than confinement in bed. During the period of reaction the cough and sputum often increase, and if care is not taken to restrain them, hemoptysis may occur. Only one case out of more than 100 cases that I treated suffered from severe pulmonary hemorrhage.

[2] Takano, R., *J. Exp. Med.*, 1916, xxiv, 207.

Case Reports.

Case 1.—Male; age 43 years. Average build. Family history negative. Infiltration of the upper lobe of the right lung. Pulmonary hemorrhage occurred in July, 1914. A diagnosis of apical tuberculosis was made. The patient has complained of coughing and sputum since Feb., 1915. Temperature 37.5°C. Pulmonary hemorrhage of about 200 cc. took place 2 weeks before admission. Nutrition fair. Anemic. Apex of the right lung dull; medium râles were heard in the same region. 7.5 cc. of cyanocuprol were given at the first injection, and in the evening a pulmonary hemorrhage of about 50 cc. occurred. For 10 days the patient expectorated bloody sputum. After an interval of 2 weeks a second injection of 7.5 cc. was given. This time he presented no ill effects, and rarely expectorated bloody sputum. After five injections he returned to his home. At that time the regional dullness had diminished, and the râles had disappeared. The bacilli in the sputum, which before treatment had been equal to Gaffky's No. VIII, were reduced to No. IV or No. V. The sputum decreased considerably. The patient gained 3.6 kilos in weight.

In one case a severe reaction occurred which resulted in an aggravation of the general symptoms.

Case 2.—Female; age 21 years. Average build. Family history positive. Suffered from infiltration of the left upper lobe of the lung. Right apical tuberculosis. Has always been healthy; no serious illness. The patient suffered from bronchial catarrh, which soon subsided and left no subjective symptoms. Nutrition fair. Face pale. Temperature normal. Pulse more than 90. On the back a dull sound was heard from the apex down to the second intercostal space covering the upper half of the left side of the interscapular region. The right side of the interscapular region had also a slight dull sound. At the left apex a number of consonant râles were heard, and at the right apex a few small râles. 7.5 cc. of cyanocuprol were given at the first injection. On the day of the injection the body temperature rose to 38.1°C. The next evening it remained at 38.4°C., and fell on the 3rd day to 37.4°C. The patient left the hospital against the advice of the physicians in charge. She reported that she had had a temperature of 37.9°C., which became normal in about 2 weeks. No change was observed in the râles and dull sound, but she complained of the sputum which did not occur before the injection. Blood streaks were observed several times in the sputum. The second injection of 7 cc. was made in 2 weeks. This time the dull sound and râles increased slightly, and on the following day the temperature rose as high as 39°C. It was preceded by shivering but no chill. The patient was dismissed on the 3rd day after the injection Every evening the temperature rose to 38.3–38.4°C., and in about a week pneumonic symptoms developed. The temperature rose as high as 39.4°C. The dull sound and the râles appeared over the whole region of the left lung. These symptoms prevented my attempting further

treatment with cyanocuprol. The patient suffered from extreme emaciation and died in about 3 months.

In this case failure resulted partly from oversensitiveness to the preparation, but chiefly from the refusal of the patient to remain quiet during the period of pronounced reaction, and also to lack of the necessary interval between the injections.

In one case severe pain was caused by the injection.

Case 3.—Male; age 43 years. Average build. Family history negative. The patient was suffering from infiltration of the right upper lobe and left apex, together with laryngeal and intestinal (?) tuberculosis. Has always been healthy; no serious illness. Since Jan., 1915, he has complained of harshness of the voice, with pain in the throat; not conscious of fever. For the past several days he has been suffering from diarrhea,—four or five stools a day,—coughing, and sputum. Appetite poor. Nutrition poor. Severe anemia. Temperature 38°C. Pulse 90. Dull sound over the region of the right apex down to the second intercostal space in front and the lower corner of scapula on the back, from the left apex to the supraclavicular fossa in front and over the corresponding region of the back. Large and small râles heard over this entire region. General respiration sound weak. The bowels are sunken. In the right iliac cavity a hard mass as large as a pigeon's egg is felt, which is not tender. Von Pirquet reaction slightly positive. After the first injection of 6 cc. of cyanocuprol the temperature reached 38.8°C. No change was observed in the lungs and intestines. The pain in the throat subsided for a short time, but reappeared on the 5th day. This time the pain was more severe, and difficulty in swallowing developed. In consequence of severe emaciation the treatment was suspended.

Koga[1] reports a case of intestinal tuberculosis that developed symptoms resembling dysentery in consequence of the injection of cyanocuprol. I have had a similar case.

Case 4.—Female; age 34 years. Average build. Mother died of pleurisy. The patient has the progressive form of pulmonary tuberculosis. Had been healthy until she contracted pneumonia in 1913. In 1914 had pleurisy of the left side, and in Mar., 1915, serous pleurisy of the right side. She catches cold easily, and has complained of harshness of the voice since Mar., 1915, with large quantities of sputum. No diarrhea. Nutrition poor. Face pale, presenting an appearance of cachexia. Temperature 38.2–38.3°C. Pulse 108, regular, thready, and weak. Both apices had a dull sound. Many medium râles were heard at the right apex, over the frontal surface of the left lung and left interscapular region. Respiration sounds weak. The lower margin of the lungs showed no respiratory movement. No change was visible in the heart or the organs in the abdominal cavity. Von Pirquet reaction negative. 0.3 gm. of pyramidone was administered, and the

patient ordered to keep quiet. The temperature became normal in a week, when 6 cc. of cyanocuprol were given. No reaction developed, and the patient was allowed to go home. On the 6th day she ate some indigestible food and diarrhea occurred. Temperature 38°C. Pain in the bowels. On the following day she passed ten pus-like, mucous, bloody stools. No bacilli of tuberculosis or dysentery were found on bacteriological examination. The symptoms subsided in a week, the temperature falling gradually to 37.4°C. In the 2nd week after the first injection she was again admitted to the hospital and 5 cc. of cyanocuprol were given. The temperature, which remained at 37.8°C. before the injection, fell to 37.4°C. on the 4th day after the injection, perhaps in consequence of keeping quiet. After the third injection a severe angina developed and the temperature rose above 39°C. Appetite poor. A fourth injection of 4 cc. was given, and on the 7th day severe cerebral symptoms developed. Patellar reflex normal. Neck not stiff. Kernig's sign absent. The patient died on the 12th day after the fourth injection.

The intensification of the symptoms is not wholly due to the effect of cyanocuprol, but I have reported these three cases in order to show that failures may be encountered. The reactions sometimes appear after a week's delay. In case of such an abnormal reaction, special precautions should be taken. The severe cases and cases suffering from progressive lesions or with a poor prognosis seem especially to suffer from retarded reactions.

Care of the Patient.—Cyanocuprol brings about an improvement in the condition when adequate doses are administered; but this alone is not sufficient, for the condition may be intensified unless special care is taken. Besides the dose of the preparation, which must be regulated for each patient under treatment, care following the injection is essential. By care is meant rest. Disappointments in the past have been explained by the fact that this care had been neglected. Rest would seem to be easy to accomplish, but it is, in practice, one of the most difficult and complex conditions to meet.

It is a well known fact that the psychical condition of the patient influences disease. This is especially true in tuberculosis, for the disease itself is apt to increase the psychic sensibility.

Case 5.—Male; age 36 years. Large build. Family history negative. Pulmonary tuberculosis in the third stage, with intestinal (?) tuberculosis. Contracted pleurisy 8 years ago. Suffered from tuberculosis of the left lung 5 years ago, which gradually spread. In 1914 his case was diagnosed at the hospital

of the Tokyo Imperial University as probable intestinal tuberculosis. Nutrition poor. Slightly anemic. Pulse more than 85, regular. Temperature 38°C. Chest poorly developed. Respiration impaired. Dull sound over the left lung, except the lower lateral part where the sound is nearly normal. Many large and small râles are heard over this region and over the right apex and the right side. Wintrich's phenomenon present at the left subclavicular region. A hardening the size of a pigeon's egg, and slightly tender, is felt at the right iliac region. The first three injections of 5 cc. each were made every other week. The râles decreased and the intestines became less hard. The patient thought he had improved somewhat. 5.75 cc. were given at the fourth injection. On the day before the injection, he received news of the death of his elder sister. In consequence of this, severe emaciation developed and he became utterly helpless. A fifth injection of 5 cc. and a sixth of 4 cc. had no effect, and the administration of cyanocuprol was abandoned.

Case 6.—Female; age 22 years. Small build. Family history negative. Progressive form of pulmonary tuberculosis in the third stage. In her 15th year she was operated on for cervical lymphangitis. Has had no other serious disease. In Feb., 1915, she caught cold and has since suffered from coughing and expectoration. Since April she has been conscious of fever, with sudden increase of coughing and expectoration. Nutrition impaired. Moderately severe anemia. Subcutaneous fat diminished. Skin loose. Pulse more than 114, small, regular, thready, and weak. Cervical glands not palpable. Chest long and narrow. Respiration normal. From both apices to the first intercostal space as well as on both sides of the chest to the fifth rib a dull sound is heard. Throughout the lungs râles are heard, mingled with consonant râles. The lower margin of the lungs has weak respiratory movements. No change is noticed in the heart and various organs in the abdomen. Temperature 38–39°C. Body weight 34.7 kilos. 5 cc. were given for the first injection. No reaction was produced. 5 cc. were given for the second injection, which again produced no reaction. By this time decrease of rales was observed and the temperature became lower. The patient felt better. 4.5 cc. were given for the third injection, after which she ejected bloody sputum. Some members of her family told her about her hopeless condition which distressed her greatly. She was not able to sit up in bed, although by the 5th day after the injection the sputum was no longer bloody. Two more injections were made, but emaciation developed rapidly and she died soon after.

That untoward psychical influences act unfavorably upon tuberculosis has been shown in many cases. Patients who suffer from hypersusceptibility are difficult to treat, and special care must be used in the administration of cyanocuprol.

Rest for the Body.—During the reaction period, the body must be kept absolutely quiet, and the patient must not be allowed to move

about, or severe pneumonia may sometimes develop, as in Case 2. External excitement causes severe reactions which interfere with improvement. Hence while under treatment the patient must be kept under the special care of the physician in the hospital. In the Kitasato Institute the patients are ordered to rest as completely as in relapse in enteric fever. The period of rest varies according to the severity of the disease and other conditions of the patient. For example, the requirement is 3 days for a patient having pulmonary tuberculosis in the first stage, 5 days for the second stage, and 7 days for the third stage. If, however, the patient is suffering from fever, hemorrhage, progressive lesion, retarded reaction, pleurisy, peritonitis, and especially from kidney tuberculosis, the period is considerably prolonged. Even after the expiration of the set period of rest, it is better to keep quiet for another period in order to avoid all danger of fever. Fever or hemorrhage may develop in consequence of a trip by railroad as well as from exercise. The greatest care is demanded at the first and the second injections and no exercise permitted.

Rest for the Lesion.—Rest for the lesion alone produces improvement and, therefore, the splint or plaster bandage is extensively applied for bone or joint tuberculosis. Artificial pneumothorax may also accomplish a similar purpose. Rest for the lesion is an essential element in bringing about complete action of the remedy, and is no less important than rest for the body. The manner of giving rest to the lesion must differ with the form of the disease. The following methods may be indicated.

Pulmonary Tuberculosis.—Coughing and speaking must be stopped. Above all, the cough must be carefully treated, for an increase of the cough often involves danger. Coughing may be satisfactorily controlled by codeine hydrochloride or similar sedatives. To remove the difficulty of expectoration in consequence of tenacity of the sputum, or in nervous coughs, the bronchitis kettle will prove valuable. I usually treat these cases with a solution containing 1 gm. of sodium bicarbonate, 1 gm. of sodium chloride, and 100 cc. of distilled water, by means of a nasal atomizer used by the patient when the coughing returns. Dust must be excluded from the air breathed. Smoking and loud or long speaking must also be prohibited.

Pleurisy.—Breathing is comparatively less harmful in pulmonary tuberculosis, but in pleurisy the movements of the chest rub the lesion. This may cause the greater reaction and less efficacy of the preparation in pleurisy than in pulmonary tuberculosis. In pleurisy, therefore, the patient must not only follow all the necessary precautions required for pulmonary tuberculosis, but the period of rest for the body must be considerably prolonged.

Laryngeal Tuberculosis.—Speaking must be forbidden in cases in which hoarseness exists. Coughing also is more harmful in this condition than in pulmonary cases. Irritating food and smoking must be abstained from.

Intestinal and Peritoneal Tuberculosis.—The peristaltic contractions of the intestine necessarily interfere with the rest for the lesion in intestinal tuberculosis. At first severe reactions were obtained in tuberculosis of the bowels, but these inconveniences have now been eliminated. The precautions taken are briefly as follows: On the day before the injection a laxative such as magnesium sulphate is administered, and on the morning of the injection an enema of saline solution is given in order to evacuate the contents of the bowels. A comparatively less amount of cyanocuprol is then injected. If the patient does not have a stool the next day, an enema or some other treatment should be given in order to insure a daily stool. After the injection no food is to be taken that may produce diarrhea or increase fermentation, and stimulating food accessories or indigestible foods are omitted. Morphine or its derivatives may be resorted to in order to control peristalsis, but they usually cause constipation and consequently incur fermentation, thus leading to stimulation of the intestinal lesions. When a severe enteric reaction arises producing symptoms resembling acute dysentery, morphine may be administered.

Surgical Tuberculosis.—For cutaneous or glandular tuberculosis, no special precaution is necessarily taken except that the lesion should not be exposed to outward stimulation. For bone or joint tuberculosis, a splint or plaster bandage should be applied.

Kidney Tuberculosis.—Similar precautions to those taken in acute nephritis are recommended. For example, stimulating foodstuffs should be strictly prohibited and the patient should be ordered to

take absolute rest. Thus far only a few cases of kidney tuberculosis have been treated.

It is an interesting fact that an intimate correlation between the doses and the rest for the lesion seems to exist. The doses given above have been established by experience. For example, small doses are prescribed in pleurisy, intestinal tuberculosis, and laryngeal tuberculosis; while in surgical cases larger doses are given. The results of the treatment also seem better in the latter than in the former conditions. The difference may possibly be accounted for by the fact that in the former cases, the drug produces severe lesional stimulation which interferes with the improvement. The only practical way of eliminating these unfavorable influences and of establishing improvement is by securing rest for the lesion.

Contraindications.—Koga points out that apricot juice must not be given with cyanocuprol, because of the increase of hydrocyanic acid, while potassium iodide, creosote, guaiacol, and their derivatives, serve to produce severe reactions, and hence all are contraindicated. It is well known that potassium iodide exerts an influence upon tuberculous lesions, for it causes inflammation at the site of the lesion. The precise action of creosote and guaiacol is not yet determined, but it is known that their hypodermic or intravenous administration produces sudden increase of the lesional reaction. All remedies which produce lesional inflammatory effects must be avoided.

Can tuberculin be given coincidentally with cyanocuprol? Koga reports that in a patient that had been treated with tuberculin, cyanocuprol produced a remarkable improvement, while the reactions had been slighter than in patients who had not had tuberculin. But it is better to avoid tuberculin while cyanocuprol is being administered, for the lesion will become sensitive to the various influences. The precise doses of tuberculin and cyanocuprol are not easily established and the combined treatment is not advised. If, however, cyanocuprol should be ineffective, tuberculin may be resorted to after a considerable interval.

The following case is of interest, as it had previously been treated with iodol.

Case 7.—Male; age 49 years. Large build. Mother and wife died of pulmonary tuberculosis. Pleurisy on both sides. Both apices infiltrated. He

has always been healthy and has never had any serious illness. Has been suffering from pleurisy since last year. Temperature had been about 39°C. at first. At present it is nearly normal. Heart frequency increased, and weakness set in. Appetite and nutrition impaired. Constipated. Color pale. Pulse more than 80, regular, medium tension. Dull sound from the apex of the right lung to the first rib in front, and the same region on the back, and at the side of the apex of the left lung. The lower lobes of both lungs have also a dull sound on the back and front. Breath sounds weak throughout. Voice vibrating and weak. On the frontal side of the lungs râles are heard. In the upper lobes on both sides of both lungs bronchial sounds are heard. 5 cc. of cyanocuprol were given at the first injection. Temperature rose to 37.7°C. The next evening it rose to 38.4°C. On the 3rd day it rose to 39.1°C. No unfavorable symptoms were observed in the chest, but the patient complained of pain in the muscles of both hips. Two hard swellings the size of hen's eggs were observed on the left side and one on the right. They were tender and felt like gristle. The patient states that they correspond to the injection points of iodol, which was given 2 months ago, and that they showed no change before cyanocuprol was given. On the 7th day the temperature fell below 37°C., but rose again to 38°C. A second injection of 4 cc. was made 3 weeks after the first. No reaction was produced and the temperature remained below 37.1°C. A third injection of 4 cc. was given 2 weeks after the second. It produced no reaction. The swellings on the hip became smaller,—no larger than a nut. They remained unchanged by the injection of cyanocuprol, and the tenderness disappeared. Symptoms of pleurisy gradually improved. The rise of temperature after the first injection seems to indicate that an intimate relation exists between the two substances.

All the agents used in symptomatic treatment, such as pyramidone, aspirin, codeine, morphine, bismuth chloride, tannigen and its derivatives, ergotine and its derivatives, digitalis and its derivatives, digestive adjuvants, etc., may be given with cyanocuprol. A general diet may also be given except in the cases mentioned.

Prognosis.—The fundamental criteria on which prognosis is based are the same with the cyanocuprol treatment as with other agents. Patients with a noteworthy family history or those in whom there is no von Pirquet reaction except when treated with tuberculin have, as a rule, a bad prognosis. The older lesions apparently are slow to react to the drug. Age seems also to interfere with its action. An acute progressive form of the disease must be treated with special precautions. Even when applied with the utmost care patients suffering from acute lesions have sometimes seemed not to do well under the treatment.

Case 8.—Male; age 37 years. Large build. Family history negative. Progressive form of pulmonary tuberculosis in the first stage. Has always been healthy; no serious illness. Contracted bronchitis in Oct., 1914, from which he recovered in Feb., 1915. In the same month he complained of recurrent bronchitis. Since then he has had moderate coughing and expectoration and sometimes night sweats. Appetite good. One or two stools a day. Nutrition poor. Anemic. Face pale. Two or three swellings as large as peas are present in the cervical glands. The laryngeal mucous membrane is pale. Pulse more than 100, small, regular. Chest long and narrow. Breathing good. Percussion normal. On deep inspiration a few medium râles are heard in the left lung. Respiration sound generally weak. Heart and abdominal organs normal. Von Pirquet reaction slightly positive. The bacilli in the sputum are equal to Gaffky's No. I. The first injection of 7.75 cc. was made when the body temperature was 38°C. It rose to 39°C. the same afternoon. It became normal in 3 or 4 days, and the râles decreased. In 2 weeks crepitations were heard at the third rib in front and in the left scapular region. A second injection of the same dose was given. This time no fever was produced. In 2 weeks, however, the crepitations in the right side of the chest changed to consonant râles. Those on the right side developed moderate consonant râles. A moderate number of râles appeared on both sides. The same dose was given at the third injection. No reaction appeared immediately, but in a week the temperature began to rise and reached 39°C. in 12 days. Râles also increased. At the right apex a dull sound was heard. In 18 days the temperature fell to 38°C. On the 25th day after the last injection the patient received a fourth injection of 6.5 cc. No rise of temperature was observed, but the râles increased. In 2 weeks a fifth injection of 5 cc. was given, and the temperature began to rise. On the 4th day it was 39.4°C. In 2 weeks a sixth injection of 4 cc. was given The temperature fell this time in a week to 38.3°C., and again rose to 39.5°C. Edema developed on the face and dorsal part of the feet. A slight anesthesia developed at the lips, abdomen, and legs, with slight tenderness in the calves. Heart normal. He probably suffered from beri-beri. A seventh injection of 3.5 cc. was given. No reaction occurred. The symptoms of beri-beri disappeared after 2 weeks of a rice-free diet and treatment with cascara and magnesium sulphate. An eighth injection of 3 cc., a ninth of 2.5 cc., and a tenth of 2.5 cc. were given. Each time no reaction occurred. The râles decreased markedly after the sixth injection. A slight dull sound appeared at the left apex after the seventh injection. Great weakness developed. The patient is still under treatment.

Idiosyncrasy and Accumulative Effect.—A severe case is generally highly susceptible to cyanocuprol. Patients in whom it is difficult to keep the lesion quiet or nervous patients sometimes suffer from severe reactions. There are still others in whom no cause for the

reaction can be accounted for. I have not yet enough data to decide whether they comprise an idiosyncrasy or not.

After the injection of cyanocuprol the lesion becomes susceptible to irritation, but on repetition of the injection no increase in the irritation has been noted. In other words, no case has been met with in which an accumulative effect of cyanocuprol was observed.

Cases in Which Cyanocuprol Did Not Lead to Improvement.

Case 9.—Male; age 33 years. Average build. Family history negative. Infiltration of the left upper lobe; tuberculosis of the right upper and lower lobes. Has always been healthy except for pleurisy in his 16th year and epidemic cerebrospinal meningitis in his 28th year. Coughing for about 1 year. Night sweats. Conscious of fever. Nutrition fair. Face pale. Pulse regular, tension normal. Chest long and narrow. Respiration good. Dull sound present from the apex of the left lung to the second intercostal space in front and the middle of the scapula. Moderate non-consonant râles from the apex of the left lung to the middle of the scapula and over the surface of the left lung in front. The same kind of râles heard over the region corresponding to the upper lobe of the right lung. Maximum temperature 37.2°C. Body weight 49.9 kilos. 7.5 cc. were given at the first injection. 2 weeks later 7.75 cc. were injected. After the second injection many tubercle bacilli were present in the sputum. Sputum has been ejected the same number of times daily as before; *i.e.*, two or three times. Since then 7 to 8 cc. of cyanocuprol have been injected every other week, but no ill effects have been observed. The only change in the symptoms has been a slight decrease of the dull sound in the upper lobe of the left lung, while a slight dull sound has developed at the apex of the left lung and the râles in the left lung have decreased slightly. Tubercle bacilli in the sputum range between 0 to No. VIII of Gaffky's table. A slight rise of temperature was observed after the seventh injection, 37.5°C., rarely 38°C. In this case cyanocuprol had no effect. Body weight lost 3.8 kilos. The patient says he has lost in body weight every summer.

Case 10.—Male; age 33 years. Average build. Had seven brothers and sisters, two of whom died of pulmonary tuberculosis. Pulmonary tuberculosis in the first stage. Has never been strong, but has had no serious illness. In July, 1914, he had hemoptysis. In Jan. and Feb., 1915, the sputum was again bloody. Temperature normal. Sleep interrupted. Appeared generally nervous. Cough and expectoration moderate. Appetite impaired. Nutrition fair. Face pale. Pulse regular, more than 85. Chest moderately developed. Respiration good. No swelling present in the cervical glands. No abnormal dull sound is heard in the chest. A few small râles are heard at the lower margins of both lungs. No abnormal symptoms are present in the heart and the organs

in the abdominal cavity. Von Pirquet reaction weakly positive. Tubercle bacilli in the sputum correspond to Gaffky's No. VIII. 7.5 cc. of cyanocuprol were given at the first injection. A slight increase in the râles was observed the next day, which decreased on the 3rd day. In 2 weeks they could be heard only when he coughed. 7.5 cc. of cyanocuprol were given at the second injection. No reaction. 7.5 cc. were given at the third injection. A slight decrease was observed in the râles which were heard at the time of coughing. No reaction present. For the fourth, fifth, and sixth injections 7.5, 7.25, and 7 cc. were given, respectively. No reactions produced. The bacilli in the sputum decreased to Gaffky's No. II to No. IV. They could not be eliminated. The râles also never subsided. Sleep was interrupted as before. The body weight decreased 2.6 kilos.

Case 11.—Male; age 22 years. Tall and thin. Mother died of pulmonary tuberculosis accompanied by intestinal tuberculosis, and one younger brother died of pulmonary tuberculosis. Pulmonary tuberculosis in the second stage, and intestinal tuberculosis. Has had no serious illness, but is subject to gastro-intestinal disturbances. For 20 days has felt feverish and had night sweats. Coughing and expectoration slight. Appetite impaired. Great emaciation. Face and mucous membrane of the mouth and the eyelids highly anemic. Chest long and narrow. Respiration good. The upper lobes of the right and left lungs have medium râles. Crepitation is heard at the lower part of the right lung. Abdomen is a little sunken. A hard mass as large as a pigeon's egg is felt at the cecum. The region around the navel is tender to the touch. Temperature 37.3°C. Pulse over 90. Body weight 41.8 kilos. The first, second, and third injections consisted of 7, 7, and 6.75 cc., respectively. After the third injection the râles in the left apex increased. The fourth, fifth, sixth, and seventh injections consisted of 7, 7, 6, and 6 cc., respectively. After the fifth injection the temperature rose to 37.5°C., but after that no rise was observed. Once after bathing it rose to 38°C., and at another time after he had eaten ice-cream when he was out of the hospital, it rose to 38.6°C. On another occasion he ate ice-cream while in bed in the hospital and the temperature rose as high as 38°C. For 9 days after the 4th day after the third injection, it ranged between 37.4–38.3°C. At the time of the seventh injection all the râles disappeared except those in the right apex. The hard mass as well as the tenderness around the cecum also disappeared. Dullness remained the same. Diarrhea remained the same as before the treatment; two or three stools daily. Emaciation developed gradually. Body weight lost 6.5 kilos. Bacilli had not been observed in the sputum at the beginning of the treatment but they became visible after the fourth injection and never disappeared. After the seventh injection he was subjected to dietetic treatment and cyanocuprol was suspended, but in spite of the effort to gain in strength, there was no improvement. The temperature remained about 38°C.

Cases in Which Cyanocuprol Produced Some Improvement.

Case 12.—Female; age 23 years. Family history negative. Infiltration of the apex of the left lung. Has been healthy. In 19th year contracted inflammation of the internal ear. 2 years ago she says she contracted pleurisy and intestinal tuberculosis. In Oct., 1914, she contracted tuberculosis of the apex of the right lung. Moderate coughing and expectoration. Occasional night sweats. Temperature 37.3°C. Nutrition fair. Color good. Pulse regular and normal. No swellings present in the cervical glands. Heart and organs in the abdominal cavity normal. Both tonsils enlarged. Mucous membrane of the larynx flushed. Spine curved to the left. Chest medium. Respiration good. A slight dull sound and a few small râles are heard throughout the left lung. Respiration weak at the lower margin of the left lung. The bacilli in the sputum are equal to Gaffky's No. III. Body weight 50.5 kilos. Von Pirquet test strongly positive. 7.5 cc. of cyanocuprol were given at the first injection. The temperature rose to 37.6°C. Coughing increased considerably. The temperature remained at 37.5°C. The same dose was given for the second, third, and fourth injections. No reaction was produced. The temperature fell to 37.3°C. after the second injection. 7.75 cc. were given for the fifth injection. This time slight fever was produced, for it remained at 37.7°C. for about a week, after which it again fell below 37°C. All the symptoms in the lungs decreased markedly after the first injection. Nearly all the dull sound as well as the râles disappeared. Expectoration disappeared after the first injection. No sputum could be obtained for bacteriological examination. Occasional inflammation of the tonsils, but no other complaints. Body weight lost 0.9 kilo.

Case 13.—Male; age 29 years. Average build. Father died of an intestinal disease. Two brothers died of meningitis. One maternal uncle died of tuberculosis. Infiltration of the upper lobes of both lungs. Patient has always been healthy except for pleurisy in his 18th year. Had pulmonary hemorrhage 4 years ago and another attack 4 months ago. At present he coughs and expectorates a little. Color good. No swelling occurs in the cervical glands. Pulse, heart, and organs in the abdominal cavity normal. Temperature normal. Chest poorly developed. Respiration weak. A slight dull sound is heard over the region from the apex down to the first rib in front and the middle of the scapula on the back. Medium râles are heard along the left lung. At the lower part of the left lung a slight dull sound is heard. The bacilli in the sputum correspond to Gaffky's No. III. Body weight 51 kilos. Von Pirquet test weakly positive. 7.75 cc. of cyanocuprol were given for the first, second, and third injections. 7.75 cc. were given for the fourth and the fifth injections, respectively. A slight increase in the râles was observed after the first, third, and fifth injections, and decrease of the dull sound was noticed. The latter remained to a slight degree on the back of both lungs and the apex of the right lung. The râles also nearly disappeared, remaining only on the back of the right lung.

The bacilli in the sputum became so few that in the ordinary film preparation none could be detected. Body weight gained 0.5 kilo.

Case 14.—Male; age 25 years. Father died of tuberculosis. Pulmonary tuberculosis in the third stage. Caries of the fifth rib and tuberculosis of the cervical glands. Has been healthy. In 7th year contracted diphtheria. In 21st year contracted tuberculosis of the cervical glands, and consequently developed anesthesia of both ears. 3 years ago remittent fever developed, ranging between 37 and 39°C. At the same time coughing and expectoration appeared. In April of last year had three hemoptyses, each time losing about 100 cc. of blood. Emaciation. At present the temperature is 37.5–38°C. Occasional hemoptyses. Moderate coughing and expectoration. Emaciation becoming more severe. Von Pirquet test negative. Nutrition poor. Face pale. Lips flushed. Swellings as large as the tip of the little finger present in the cervical glands on both sides. Scars at the left side of the neck and over the fifth costal cartilage on the right are both due to an operation. Closed pustulation as large as a pigeon's egg present at the lower frontal part of the sternum. Pulse more than 90, regular, tension slightly decreased. Chest narrow, especially on the left side. Dull sound over the surface of the left lung. From the apex to the second intercostal space on the right frontal side and the lower end of the scapula on the back is heard a slightly dull sound. Over the whole region where the dull sound prevails and at the region corresponding to the lower lobe of the right lung consonant large and medium râles are heard. Dimension of heart could not be determined. Heart sound normal. Abdomen normal. Body weight 45 kilos. 6.5 cc. were given at the first injection. The following injections were made every other week, each dose being 5 cc. No ill effects appeared. After the fourth injection the temperature did not rise above 37.4°C. The general condition of the patient improved greatly. The dull sound as well as the râles became considerably less. The bacilli in the sputum, which corresponded to Gaffky's No. VIII, never exceeded Gaffky's 0 to No. III. The body weight gained 0.3 kilo.

Case 15.—Female; age 17 years. Average build. An elder sister died of pulmonary tuberculosis. Pulmonary tuberculosis in the third stage, acute, progressive form. Has always been healthy. A year ago suffered from coughing and expectoration, the temperature reaching sometimes as high as 38–39°C., which always subsided in several days. A similar attack occurred 9 months ago and was diagnosed as pleurisy. Severe coughing and night sweats occurred. Nutrition fair. Face pale. Lips cyanotic. Mucous membrane of the eyelids and mouth pale. No swelling present in the cervical glands. Pulse small, regular, and over 90. Chest long and narrow. Respiration weak. The heart beat is conspicuous at the second and third intercostal space. Dull sounds are heard over the surface of the right frontal side of the lung to the second intercostal space on the left and to the lower edge of the right scapula, and over nearly all the surface of the right lung on the back. Many large and medium râles are heard over the whole sur-

face of both lungs except the lower dorsal part. The heart is normal except for a slight hemic murmur. Abdominal organs all normal. Temperature is 38–39°C. Body weight 37.5 kilos. 6.5 cc. of cyanocuprol were given at the first injection. It produced a local reaction. A second injection of 6 cc. and a third of 6.25 cc. were given. 2 or 3 hours after the third injection the temperature rose to 38.4°C., which soon subsided and remained below 37°C. All the symptoms improved considerably. At the fourth, fifth, sixth, and seventh injections, the doses were 6.25 cc. After the fourth injection the temperature did not rise above 37.4°C. Night sweats practically subsided. Severe pathological processes completely checked. At the eighth and ninth injections 6.5 cc. were given. The dull sound and the râles had become considerably less. After the ninth injection the temperature rose to 37.7°C. and resulted in a slight increase of the dull sound and râles. For the tenth injection 5 cc. were given. The patient is still being treated.

Case 16.—Male; age 20 years. Family history negative. Infiltration of the upper lobe of the right lung and peritoneal tuberculosis. In 13th year contracted pleurisy. In 17th year had a little bloody sputum. For 4 months has been complaining of general dullness. Some months ago swelling of the abdomen and pain in the region of the stomach developed. He has been conscious of fever several times a day. No coughing or expectoration. Appetite normal. Stools once every other day; occasional diarrhea. Respiration labored and deep. Nutrition good. Not anemic. Pulse regular and normal. Chest well developed. Slight dull sound is heard at the upper lobe of the right lung. At the right lateral upper part is heard a clear bronchial respiration sound. Many medium râles at the right apex. The right axillary cavity has a dull sound and a few crepitations. Respiration normal at the lower edges of the lungs. Abdomen swollen; right side resistant. In the right iliac cavity is a tender hard mass as large as a pigeon's egg. No exudate is present in the abdominal cavity. Temperature 37.3°C. Body weight 60.1 kilos. 7.75 cc. of cyanocuprol were given at the first injection. On the following day suffered from dizziness; temperature rose to 39.4°C. It fell to the usual point the next day. This is one of the highest febrile reactions that I observed, the temperature varying 2.1°C. A second injection of 7.75 cc., a third of 7.75 cc., and a fourth of 7.5 cc. were given; no reaction followed. After the first injection, the resistance of the abdomen disappeared rapidly. The dull sound of the lung also disappeared, and the râles became fewer. At the fourth injection the symptoms of the lungs and the resistance of the abdominal wall had disappeared completely. The patient did not follow the prescribed diet, and the hard mass in the right iliac cavity became more prominent. He suffered from diarrhea. Body weight lost 1.2 kilos.

Case 17.—Female; age 33 years. Husband suffering from a light case of pulmonary tuberculosis. Caries of the sixth dorsal and the fourth lumbar vertebræ. Infiltration of the apex of the right lung. Patient has always been healthy; no noteworthy illness. 9 months ago she began to complain of general dullness

and occasional fever. No coughing or expectoration. Occasional pain in the left side of the chest. Pain in the back and hips when moving. Appetite normal. No night sweats. Nutrition fair. Slightly anemic. Pulse regular, tension good, more than 78. Right submaxillary glands slightly enlarged. Chest long and narrow. Respiration normal. A slightly dull sound is heard at the apex of the right lung. Crepitations are heard at the second and third intercostal spaces on the left side. The sixth dorsal and the fourth lumbar vertebral processes are tender. Heart and abdominal organs normal. Reflex of knee joint normal. 7.5 cc. of cyanocuprol were given at the first injection. Crepitation increased. The second, third, fourth, and fifth injections were given, the doses being the same as the first. A sixth injection of 7 cc. was given. No reaction developed. The symptoms of the lungs disappeared almost completely, except for a slight weakness of the respiratory sound. A year ago she began to suffer from hiccough, which occurred for 2 or 3 hours daily. This ceased entirely after the third injection. At the same time the pain in the chest disappeared, and there are no other symptoms. She conceived 1 month before the first injection was given. Cyanocuprol had no influence upon pregnancy in this case.

Case 18.—Female; age 28 years. Family history negative. Pulmonary tuberculosis in the second stage and intestinal tuberculosis. Pregnant 7 months. Has always been healthy. A month ago she began to suffer from coughs, fever, pain in the stomach, etc. Diarrhea six or seven times daily. No expectoration. Nutrition poor. Very anemic. Temperature 38°C. Pulse more than 90, regular, weak. Chest long and narrow. Respiration weak. No swelling in the cervical glands. The apices of the lungs and the lower side of the left lung have a slightly dull sound. A tympanic sound is heard at the side of the left apex. Numerous medium râles are heard over the side and front of the left lung. A bronchial respiration sound is heard at the upper frontal side of both lungs and the interscapular region. The abdominal region is swollen and the swollen uterus can be felt. Above the uterus is felt a movable soft mass lying longitudinally. It is slightly tender. In the right iliac cavity there is a tender region. Owing to pregnancy minute particulars could not be determined. Body weight is 42.2 kilos. 4 cc. of cyanocuprol were given at the first injection. After the injection the temperature rose as high as 38.7°C. On the following day it fell to the usual point. The patient complained of a slight pain in the abdomen. No change occurred in the symptoms of the lungs. From the 5th day after the first injection the temperature did not exceed 37.2°C. Diarrhea decreased to one or two times daily. Abdominal pain also subsided. 5 cc. were given for the second injection. No reaction occurrred. Diarrhea stopped. A third injection of 5 cc. and a fourth of 4.5 cc. were given. The temperature rose to 37.4°C., and fell to 37°C. on the 3rd day. The dull sound decreased a little but no change occurred in the râles. As labor approached the treatment was suspended. Cyanocuprol again produced no harmful effect on pregnancy. The intestinal tuberculosis improved greatly, but the pulmonary symptoms remained unimproved.

I deem it proper to state here that the cases which have improved and which ceased to receive further treatment have been examined afterwards from time to time, and thus far have rarely relapsed. Moreover, those that had some slight symptoms left when they ceased to receive cyanocuprol were found to have continued to improve without receiving further medical treatment. I have seen no case in which the symptoms increased after the completion of the treatment with cyanocuprol.

SUMMARY.

Cyanocuprol is markedly effective in tuberculosis, and we believe that it will play an important part in clinical medicine. It may be used more generally than tuberculin.

The amount of the dose is closely related to the reaction and the final results. It should be determined for each patient after a careful examination of his symptoms. The maximum dose of 8.5 cc. should in no case be exceeded.

The shortest interval between injections should be 2 weeks. If the drug is given after a shorter interval, no improvement is observed and the effects are sometimes dangerous.

In order to obtain the best results the patient should be placed under conditions of complete physical and mental rest after the injection; this applies even to light cases. Care should also be taken to secure rest for the lesion.

During the period of the treatment irritants to the lesion, such as potassium iodide or tuberculin, should be avoided; apricot juice, guaiacol and its derivatives, and iodol are contraindicated.

No marked idiosyncrasy has been noted and no accumulative effects have been observed.

I desire to express my indebtedness to Dr. Kitasato for privileges in connection with the execution of the present work and to Dr. Koga and Mr. Nemoto for suggestions and assistance.

THE TREATMENT OF LEPROSY WITH CYANOCUPROL.

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The present communication is a brief report upon the use of cyanocuprol in the treatment of leprosy. Cyanocuprol has proven of value in the treatment of tuberculosis in animals and has been employed also in the treatment of human cases of the disease.[1] Hence it was concluded to test its chemotherapeutic action in leprosy. Thus far no specific treatment for leprosy has been discovered, and I am presenting this report, though brief, for it seems to contain an advance at least in the treatment of that malady.

Because of a similarity which exists between the bacilli of leprosy and tuberculosis, the attempt to apply the same curative substances both to tuberculosis and to leprosy seems reasonable. Since the experimental production of leprosy in animals has not been effected and since cultivation of the bacillus of leprosy has not yet been surely accomplished, the application of proposed curative substances must necessarily be made to human cases directly. In this instance the chemical preparation has been administered to numerous human patients on a basis of established clinical observation.

In the treatment of tuberculosis with cyanocuprol special attention was paid to the local and general reaction, because in tuberculosis an intimate relation seems to exist between the dose and the pathological process. It was anticipated that the local reaction would be of consequence in the treatment of leprosy, for the local lesions chiefly occupy the surface of the body; but contrary to my idea no such reactions occurred. Moreover, general reactions were also absent.

At first 16 to 20 mg. were given every other week. I have now come to the conclusion that 20 to 24 mg. may be given weekly without ill effects. The administration was exclusively by intravenous

[1] Koga, G., *J. Exp. Med.*, 1916, xxiv, 107, 149.

injection with an ordinary syringe, the needle being inserted into the femoral vein. The injection is made very slowly. After seven or eight injections symptoms such as congestion of the head, forcible deep breathing, acceleration of the pulse, etc., similar to what is known as anaphylactoid in salvarsan injections have been encountered. But the symptoms are fleeting, and recovery occurs in a moment without discomfort. Women are more often affected than men. These effects can, however, be obviated if the injection is made slowly enough and the patient is allowed to rest for a short time after the operation.

The following are the reports of cases of leprosy treated in the I-hai-yen Leprosorium, Meguro Village in Tokyo. More detailed reports will be made later.

Case 1.—T. H., female, aged 25 years. Lepra maculosa. No family history. In February 1914, local anesthesia and pigmentation appeared on the right arm. Confined in December of the same year. At that time a great many large and small spots appeared all over the surface of the body. I examined the patient first in April, 1915. Her face was dark purple in color with many infiltrations in the skin. Many spots as large as the tip of the little finger were scattered over the face, arms, and body, which lacked sensation. An injection of 10 mg. was given on Apr. 22. No rise of temperature occurred, or any other ill effects. A second injection of 10 mg. was given on May 10. After the injection the lesions presented slight congestion. A peculiar sensation was felt in the arms. A third injection of 8 mg. was given on May 24, after which the general symptoms seemed to improve. A fourth injection of 8 mg., a fifth of 8 mg., a sixth of 10 mg., a seventh of 12 mg., and an eighth of 12 mg. were given successively on May 28 and June 3, 10, 17, and 28. During this period the infiltration and the pigmentation of the face gradually disappeared and the skin recovered a healthy color. A ninth injection of 12 mg. was given on July 5. This time, immediately after the injection, the patient complained of congestion in her head. A still stronger congestion was experienced after the tenth injection of 12 mg. on July 12. In each instance the congestion subsided in a few minutes. An eleventh injection of 10 mg., a twelfth of 10 mg., a thirteenth of 10 mg., a fourteenth of 10 mg., and a fifteenth of 10 mg. were given successively on July 20 and 26, and Aug. 2, 9, and 16. Each time a fleeting sensation of congestion occurred.

Gradual improvement of the symptoms has been observed since the first injection. The color of the face and the consistency of the skin have become nearly normal. Sensation has also been almost completely restored. However, several anesthetic, roseola-like spots still remain in the submaxillary region. The spots on the other parts of the body have become white, and sensation has

been completely restored. Some of the spots have assumed a normal color. Nutrition good.

Case 2.—H. O., female, aged 23 years. Lepra maculosa and anæsthetica. No family history. The first symptoms appeared 3 years ago. They seemed to improve for a while but became worse during the spring of 1915. I examined the patient for the first time in May, when most of her face was covered with dark red spots. The same kind of spots was present on both arms. The left little finger became withered and could hardly be clenched. Infiltration occurred in the spot. Sensation lacking. The first injection of 10 mg. was given on May 20. No reaction occurred. The second injection of 8 mg. was given on May 29. The infiltration of the skin seemed to improve greatly. A third injection of 8 mg., a fourth of 10 mg., a fifth of 12 mg., a sixth of 12 mg., and a seventh of 12 mg. were given successively on June 3, 10, 17, and 28, and July 2. Immediately after the seventh injection the patient suffered from a fleeting congestive feeling in her head. An eighth injection of 12 mg. was given on July 12, when there was still stronger congestion. A ninth of 10 mg., a tenth of 9 mg., an eleventh of 10 mg., a twelfth of 10 mg., and a thirteenth of 11 mg. were given successively on July 20 and 26, and Aug. 2, 9, and 16. All the last five injections were accompanied by congestion in the head.

A fourteenth injection of 11 mg. and a fifteenth of 11 mg. were given on Aug. 23 and 26, respectively. In the last two injections the liquid was introduced very slowly into the vein, and no reaction occurred.

At present the facial spots are hardly noticeable. All the other spots are becoming paler, and sensation is gradually being restored. The left little finger is gradually recovering its motility, but is still withered; the tips of the fingers are becoming rounder in form and the palm is becoming thicker. Nutrition good.

Case 3.—J. Y., male, aged 17 years. Lepra maculosa and anæsthetica. One of his brothers is a leper. The symptoms appeared first in 1914. I examined the patient for the first time in May, 1915. His right hand is withered and the fingers cannot be clenched or opened. On the inner side of the joint of the right arm, a spot accompanying thickening of the skin occurred. On the inner side of the lower right arm a row of small protuberant spots was present. On the exterior side of both upper legs were a great many spots.

The first injection of 10 mg. was given on May 10, and was accompanied by no reaction. A second injection of 10 mg. was given on May 20, and a third of 8 mg. on May 28. The symptoms in the right arm improved. A fourth injection of 18 mg., a fifth of 11 mg., a sixth of 12 mg., a seventh of 12 mg., an eighth of 12 mg., and a ninth of 12 mg. were given successively on June 3, 10, 17, and 28, and July 5 and 12. After the last injection congestion of the head was felt. A tenth injection of 10 mg. was given on July 20, which produced a slight feeling of congestion in the head. An eleventh injection of 10 mg., given on July 26, was accompanied by the same reaction. A twelfth injection of 10 mg. was given on Aug. 2. The patient was allowed to lie still during the operation and no reaction occurred after the injection. A thirteenth injection of 11 mg., a

fourteenth of 11 mg., a fifteenth of 11 mg., and a sixteenth of 11 mg. were given successively on Aug. 9, 16, 23, and 26.

The infiltration of the spots in the region of the joint of the right arm has disappeared, and the pigmentation and infiltration of the small nodular spots has improved. There appears little or no trace of leprous changes. One of the fingers which could not be moved recovered its motility somewhat, as well as its power of sensation. The spots on the back and legs are becoming whiter in color and gradually disappearing. Nutrition good.

Case 4.—R. O., male, aged 35 years. Lepra maculosa and anæsthetica. No family history. The symptoms first appeared several years ago; recently new spots began to appear on the upper and lower limbs. Neurasthenia occurred occasionally. The first injection of 6 mg. was given on May 28. No reaction followed. A second injection of 10 mg. was given on July 5, when the tone of the pigment was observed to fade. A third injection of 10 mg. and a fourth of 10 mg. were given on July 12 and 20, respectively. The spots became very indistinct. After the fifth injection of 10 mg., given on July 26, congestion was felt in the head. A sixth injection of 10 mg., given on Aug. 2, was made while the patient was allowed to lie quietly in bed and no feeling of congestion in the head insued. A seventh injection of 10 mg., an eighth of 10 mg., a ninth of 10 mg., a tenth of 11 mg., and an eleventh of 10 mg. were given successively on Aug. 9, 16, 19, 23, and 26.

Both the old and new spots are fading. No neurasthenia. Sensation is gradually being restored. The patient is receiving the injection twice a week, without any unfavorable reaction. Nutrition good.

Case 5.—N. K., male, aged 28 years. Complicated leprosy. No family history. At the review of the militia in 1915, he was found to have leprosy. A slight infiltration occurred all over the face. At the right arm joint three nodules as big as peas were present. Some small subcutaneous nodules were felt on both calves. The external side of the lower left limb was anesthetic.

A first injection of 10 mg., a second of 10 mg., a third of 10 mg., and a fourth of 10 mg. were given successively on Aug. 2, 9, 16, and 23.

After the first injection the nodules in the right arm were absorbed, leaving brownish spots. By the fourth injection, two of the three spots became white and sensation was restored. The remaining one is still anesthetic. The nodules on the calves are softer, while the face is paler. The anesthesia of the lower left leg is recovering. Nutrition good.

Case 6.—K. Y., male, aged 34 years. Complicated leprosy; has pulmonary tuberculosis. No family history. Small nodules occurred all over the face. Spots were also present at the left elbow and the right knee. On the exterior side of the lower right arm, a protuberant spot was present. There were some small nodules on the calves.

The first injection of 9 mg., given on Aug. 2, was accompanied by dyspnea. The temperature rose as high as 38.5°C. This reaction occurred invariably in tuberculosis. A second injection of 9 mg., a third of 10 mg., and a fourth

of 10 mg. were given successively on Aug. 9, 16, and 23. The last injection caused a rise of temperature of 37.5–37.6°C.

The infiltration of the face is fading. The nodules became more conspicuous for a while, but became gradually smaller. The infiltration in the spots of the left elbow is becoming softer. The nodules of the calves are also becoming softer. The tubercular lesions are becoming very small.

I have injected cyanocuprol three times in more than ten cases and twice in more than twenty cases with old lesions. Some of them showed great improvement. A more detailed report will be published later.

SUMMARY.

This brief report deals with six cases of leprosy in which cyanocuprol has been administered with what appear to be beneficial effects. The treatment is being continued in still other cases.

A NON-GAS-PRODUCING STRAIN OF THE HOG-CHOLERA BACILLUS ISOLATED FROM AN OLD LABORATORY CULTURE.

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It is not uncommon to find minor variations among strains of the same species of bacteria, and in cultures that have been kept for several years on artificial media such changes are relatively frequent. It is seldom, however, that one finds a variation in the action on the more commonly used carbohydrates. When grown in carbohydrate media contained in the fermentation tube as recommended by Theobald Smith (1) for the determination of the action of bacteria on carbohydrates, the constancy of the action of a given species is remarkable. This method is especially valuable in the differentiation of the members of the colon-typhoid group of organisms, and a deviation from the usual action is of interest in its relation to classification and to the possible formation of new species. One of our stock cultures of the hog-cholera bacillus, an organism belonging to the paratyphoid group, has shown such a deviation in that it has lost the power to form gas from a number of carbohydrates, while acid formation remains. A review of the literature shows that non-gas-producing paratyphoid strains have been reported several times.

Dorset (2) describes an organism which he obtained from the spleen of a pig dying of acute hog-cholera. This organism was not agglutinated by the serum of a typhoid patient or by the dried serum of a guinea pig injected with this non-gas-producing organism.

Preisz (3), Bock (4), Grabert (5), and Bainbridge (6) have described cultures obtained from swine, which resembled the hog-cholera bacillus except that they failed to produce gas from dextrose.

Christiansen (7) isolated from nineteen calves non-gas-producing organisms which were agglutinated by an anti-Gaertner and an antiparacolon serum. The

latter serum protected to some extent mice injected with these organisms. After long cultivation on a variety of media he did not succeed in getting the organisms to form gas from dextrose.

Wagner (8) isolated from the blood of a woman *B. typhosus* and a non-gas-producing paratyphoid bacillus. 6 days later he isolated from the same patient a typical gas-producing paratyphoid. The last two organisms were agglutinated by an antiparatyphoid β serum and their carbohydrate reactions were the same, except that no gas was formed by the first organism.

Oette (9) and Ohno (10) have isolated from patients organisms that were agglutinated by paratyphoid β serum and that culturally corresponded to this group except that they failed to produce gas from dextrose and other carbohydrates.

It is not our purpose to review the many papers on bacterial mutations, for a review, with the literature on the subject, can be found in the work of Eisenberg (11). There are, however, a few papers that are directly related to the culture to be discussed.

Loewenthal and Seligmann (12) describe a non-gas-producing paratyphoid that they found in one of their stock cultures. This culture was isolated in 1908 in connection with the study of a meat poisoning epidemic and corresponded to paratyphoid β. For 3 years it was kept on slant agar, transfers being made every 6 to 8 weeks. In 1911 they began using it for diagnostic purposes and transfers were made two or three times a week. A year and a half later it was found that the culture no longer produced gas in dextrose or mannite media, while the original stock had retained this power. Passage through animals did not restore this lost property.

Hübener (13) found that paratyphoid bacilli grown at 46°C. lost the power to form gas from dextrose, but this power was quickly regained when the culture was again incubated at 37°C.

Penfold (14), growing paratyphoid organisms on agar plates containing sodium mono-chloracetate, obtained subcultures that failed to produce gas in dextrose and maltose media, while they retained the power to form gas from dulcite, mannite, and sorbite.

The culture on which we wish to report was received from Dr. Dinwiddie in 1899 and has been kept in stock under the name of Hog-cholera Ark. In 1901–02 Smith and Reagh (15) passed it through a series of thirteen rabbits. A bouillon culture from the heart's blood was used for each succeeding inoculation. Since that time this rabbit culture as well as the original stock has been kept on slant agar, transfers being made every 4 weeks. In 1914 the culture that had been passed through the rabbits was plated and to our surprise we found that transfers from over half the colonies studied failed to form gas in dextrose bouillon.

TABLE I.

Carbohydrates from Which Hog-Cholera Bacilli Form Gas.

Culture.	Results of titrations of fermented bouillon plus the following carbohydrates after 5 days at 37°C.											
	Dextrose.		Levulose.		Galactose.		Maltose.		Mannite.		Dulcite.	
	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.
Hog-cholera Ark., original.	Acid 4.2%	50% Gas.	Acid 4.1%	43% Gas.	Acid 3.5%	54% Gas.	Acid 4.1%	47% Gas.	Acid 2.2%	40% Gas.	Acid 0.5%	67% Gas.
Hog-cholera Ark., rabbit series, gas-forming.	Acid 4.0%	48% Gas.	Acid 4.0%	53% Gas.	Acid 3.5%	32% Gas.	Acid 3.2%	48% Gas.	Acid 1.7%	52% Gas.	Acid 3.0%	83% Gas.
Hog-cholera Ark., rabbit series, non-gas-forming.	**Acid 3.9%**	**No gas. Acid 3.8%**	**Acid 3.8%**	**No gas. Acid 3.1%**	**Acid 3.3%**	**No gas. Acid 3.0%**	**Acid 4.1%**	**No gas. Acid 4.2%**	**Acid 4.1%**	**No gas. Acid 3.9%**	**Acid 3.1%**	**No gas. Acid 3.0%**
Typhoid IX.	Acid 4.3%	Acid 4.1%	Acid 3.8%	Acid 3.9%	Acid 3.6%	Acid 3.3%						
Typhoid X.	Acid 4.4%	Acid 4.0%	Acid 4.2%	Acid 3.6%	Acid 4.0%	Acid 3.6%						

TABLE II.

Carbohydrates from Which Hog-Cholera Bacilli Do Not Form Gas.

Culture.	Results of titrations of fermented bouillon plus the following carbohydrates after 5 days at 37°C.									
	Lactose.		Saccharose.		Raffinose.		Dextrin.		Salicin.	
	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.	Bulb.	Branch.
Hog-cholera Ark., original.					Alkali.	Acid 0.7%	Alkali.	Large bubble of gas. Acid 1.6%*	Alkali.	Acid 0.4%
Hog-cholera Ark., rabbit series, gas-forming.	Acid 0.4%	Acid 1.2%	Neutral.	Acid 1.2%	Alkali.	Acid 0.7%	Alkali.	Small bubble of gas. Acid 1.3%*	Alkali.	Acid 0.5%
Hog-cholera Ark., rabbit series, non-gas-forming.	**Acid 0.5%**	**Acid 1.6%**	**Neutral.**	**Acid 1.3%**	**Alkali.**	**Acid 1.0%**	**Alkali.**	**Acid 1.6%***	**Alkali.**	**Acid 0.8%**
Typhoid IX.					Alkali.	Acid 0.7%	Alkali.	Acid 2.0%*	Alkali.	Acid 0.5%
Typhoid X.					Alkali.	Acid 1.0%	Alkali.	Acid 2.3%*	Alkali.	Acid 0.6%

* The original reaction of the bouillon was acid 0.8 per cent.

Cultural, Morphological, and Pathological Studies.

The non-gas-forming culture was compared with a gas-producing strain from the rabbit stock; with the original culture that had not been passed through the rabbits; and with two cultures of *Bacillus typhosus*. The non-gas-producing organisms from 24 hour agar and bouillon cultures cannot be differentiated morphologically from typhoid or the other strains of the hog-cholera bacillus, except that they are not quite as actively motile as the former. In 1894 Smith (16) pointed out, however, that in animals dying from an infection with the hog-cholera bacillus there was a marked morphological difference between the organisms found in tissues and those found in cultures. The non-gas-producing culture shows this same change in morphology under similar conditions, which, of course, differentiates it from the typhoid bacillus.

In bouillon it does not grow as readily as typhoid, but at the end of 5 days the turbidity of the two cultures is about the same. The carbohydrate reactions of these cultures are given in Tables I and II. It will be seen that except for the loss of power to produce gas the action on the carbohydrates is the same as that of the parent stock. It differs from the typhoid bacillus in that it fails to act on dextrin as readily as the latter, but this difference is one of degree only.

In Table III, which gives the results of titrations made on fermentation tubes containing milk, it will be seen that hog-cholera bacilli are differentiated from typhoid bacilli in that they form alkali more readily in the presence of oxygen. Here too the difference is one of degree only, for in 20 days one of the typhoid cultures formed as much alkali as the hog-cholera cultures (17).

The indol reaction of all the hog-cholera cultures tested was slight, while that of the typhoid cultures was negative.

It is interesting to note that together with the loss of power to produce gas from carbohydrates, this culture fails to decolorize neutral red dextrose agar, while the gas-forming strains decolorize this medium in 24 hours.

The agglutination tests given in Table IV sharply differentiate the non-gas-producing culture from the typhoid bacillus, but show no difference between the former and the other cultures of the hog-cholera bacillus.

TABLE III.

Growth of Hog-Cholera and Typhoid Bacilli in Fermentation Tube Containing Sterile Milk.

Culture.	After 10 days.			After 20 days.		
	Bulb.		Branch.	Bulb.		Branch.
	Condition.	Reaction.	Re-action.	Condition.	Reaction.	Re-action.
Hog-cholera Ark., original.	Clarified.*	Alkali. 0.6%	Acid 2.2%	Clarified.	Alkali 0.6%	Acid 2.2%
Hog-cholera Ark., rabbit series, gas-forming.	Clarified.	Alkali 0.6%	Acid 2.2%	Clarified.	Alkali 1.0%	Acid 2.2%
Hog-cholera Ark., rabbit series, non-gas-forming.	Clarified.	Alkali 0.6%	Acid 2.3%	Clarified	Alkali 0.8%	Acid 2.2%
Typhoid IX.	Not clarified.	Acid 0.6%	Acid 2.5%	Not clarified.	Alkali 0.3%	Acid 2.2%
Typhoid X.	Not clarified.	Acid 0.6%	Lost.	Clarified.	Alkali 0.7%	Acid 2.3%
Control tube.		Acid 1.5%	Acid 1.5%		Acid 1.5%	Acid 1.5%

* Partly translucent.

TABLE IV.

Agglutination Tests.

Culture.	Limit of agglutination.	
	Anti-typhoid serum.	Anti-hog-cholera bacillus serum.
Hog-cholera Ark., original	$\frac{1}{80}$	$\frac{1}{10,240}$
Hog-cholera Ark., rabbit series, gas-forming	$\frac{1}{80}$	$\frac{1}{10,240}$
Hog-cholera Ark., rabbit series, non-gas-forming	$\frac{1}{160}$	$\frac{1}{10,240}$
Typhoid IX.	$\frac{1}{40,960}$	$\frac{1}{80}$
Typhoid X.	$\frac{1}{40.960}$	$\frac{1}{80}$

The virulence of this non-gas-producing culture was compared with that of the gas-producing strain of the rabbit series and of the original stock by injecting known amounts of 24 hour bouillon cultures into rabbits of approximately equal weight. The results are summarized in Table V and show that the cultures passed through rabbits 15 years ago do not differ in their virulence, and are still markedly more virulent for the rabbit than the stock from which they originally came.

TABLE V.

Virulence Tests.

Culture. Hog cholera Ark.	Amount of 24 hr. bouillon culture injected into subcutis.	Weight of rabbit.	Died.
	cc.	*gm.*	*days*
Original	0.5	1,688	14
	0.1	1,406	24
Rabbit series, gas-forming	0.5	1,652	6+
	0.1	1,472	6−
Rabbit series, non-gas-forming	0.5	1,642	8−
	0.1	1.535	6−

The loss of power to produce gas from dextrose and other carbohydrates has persisted over a period of 18 months. The culture in the interval was kept on slant agar, and transfers were made monthly. Each transfer was incubated for from 24 to 48 hours and then placed in the refrigerator until the next culture was made. Rapid transfer through bouillon and transfers made directly from old agar slant growth to sugar tubes have failed to show a reversion to the original type of action. Passage through rabbits and mice has also failed to restore the power to produce gas.

DISCUSSION.

At first it was thought that the long cultivation on an artificial medium might have produced this change, but an examination of the other hog-cholera stocks showed that all the subcultures examined from colonies on agar plates produced gas in dextrose bouillon. One of these stocks had been in the laboratory for 30, another for 18 years, while three had been isolated more recently.

As the parent stock and the culture in which this new form occurred differed in that the latter had been passed through a series of rabbits, it seemed logical to assume that the animal transfers had been in some way responsible for the change. If this were the case, one should be able to produce a similar change in another strain. This was attempted by passing a recently isolated strain of the hog-cholera bacillus through a series of eleven rabbits, the inoculations being made by using a suspension of the crushed spleen of the animal dying of the disease to infect the next rabbit. The culture showed a marked increase in virulence, but no change in the gas production could be detected. From the last three rabbits in the series agar plates were made from the suspension of the crushed spleen and transfers made to dextrose Durham tubes from approximately fifty colonies in each case. All these tubes showed gas production.

Several environmental changes were tried in order to determine whether they would influence gas production. Transfers through twenty tubes of plain bouillon and bouillon containing 5 per cent of sodium chloride failed to influence gas production. Transfers through bouillon containing anti-hog-cholera bacillus serum were also without effect. Cultivation for $3\frac{1}{2}$ months in Dunham's peptone solution failed to yield cultures that differed from the original culture in their action on dextrose.

The negative results of attempts to influence gas production by means of environmental changes show that we are dealing with a highly stable property. The failure to influence it by the animal passages may mean that we selected a more resistant strain than the Hog-cholera Ark., or it may mean that the rabbit passages had nothing to do with the change that occurred in the latter culture. The possibility of this new form being a contamination can be ruled

out, for we have not had such a culture among our laboratory stocks; and the finding of paratyphoid bacilli in spontaneous diseases of rabbits has not to my knowledge been reported.

The loss or perhaps gain of cultural characters in a given strain is very different from the transformation of one species of bacteria into another, as has been recently reported by M'Gowan. In association with Wang (18), M'Gowan reports the transformation of *B. avisepticus* (fowl-cholera bacillus) by passing it through a series of fourteen guinea pigs, into a motile organism that forms acid and gas from a number of carbohydrates including lactose and saccharose. The serum of an animal immune to this motile organism did not agglutinate the original culture from which it is supposed to have come. In spite of the fact that this motile organism resembles *B. coli* more than any other, M'Gowan makes certain assumptions based on this transformation and the fact that in his hands members of the septicemia hemorrhagica group have shown marked variability in their cultural reactions. In discussing swine fever (19) he assumes that *B. suipestifer* (hog-cholera bacillus) is a variant of *B. suisepticus*, and in his paper on fowl-cholera (20) he assumes that *B. pullorum* and the fowl typhoid bacillus are variants of *B. avisepticus*.

These assumptions might explain many puzzling questions, but the facts on which they are based do not agree with our experience. Using the fermentation tube, as recommended by Smith (1) in determining the sugar reactions, members of the paratyphoid and septicemia hemorrhagica groups have shown marked stability in their cultural characters. There are essential differences between the cultural reactions of these two groups, the type of disease they produce when injected into animals is entirely different, and they have shown no cross immune reactions. Such transformations as M'Gowan reports must therefore be looked upon with doubt until we have more definite proof that they occur.

SUMMARY.

In a stock culture of the hog-cholera bacillus, which was passed through a series of rabbits 14 years ago, an organism was found that differs from the original culture in that it fails to form gas from the carbohydrates that are usually attacked by this organism, while acid formation persists. This new strain is agglutinated by an anti-hog-cholera bacillus serum and produces in rabbits and mice a disease

similar to that caused by the typical cultures. The failure to form gas has persisted over a period of 18 months and all attempts to cause the strain to revert to the original condition have failed. It resembles in many respects *Bacillus typhosus* and it may be that some of the so called typhoid cultures that are not agglutinated by antityphoid serum are non-gas-producing paratyphoids. Attempts to produce a similar change in a more recently isolated culture of the hog-cholera bacillus by means of animal passages and changes in the environment have been negative.

BIBLIOGRAPHY.

1. Smith, T., *Centr. Bakteriol.*, 1890, vii, 502; *Am. J. Med. Sc.*, 1895, cx, 283.
2. Dorset, M., *U. S. Dept. of Agriculture, Bureau of Animal Industry, 18th Ann. Rep.*, 1901, 566.
3. Preisz, H., *Z. Thiermed.*, 1898, ii, 1.
4. Bock, F., *Arb. k. Gesndhtsamte.*, 1906, xxiv, 238.
5. Grabert, K., *Z. Infektionskrankh. Haustiere*, 1908, iii, 218.
6. Bainbridge, F. A., *J. Path. and Bacteriol.*, 1909, xiii, 443.
7. Christiansen, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1914, lxxiv, 474.
8. Wagner, G., *Centr. Bakteriol., 1te Abt., Orig.*, 1913, lxxi, 25.
9. Oette, E., *Centr. Bakteriol., 1te Abt., Orig.*, 1913, lxviii, 1.
10. Ohno, K., *Centr. Bakteriol., 1te Abt., Orig.*, 1915, lxxv, 288.
11. Eisenberg, P., *Ergenbn. Immunitätsforsch.*, 1914, i, 28.
12. Loewenthal, W., and Seligmann, E., *Berl. klin. Woch.*, 1913, l, 250.
13. Hübener, E., quoted from Uhlenhuth, P., and Hübener, E., Kolle, W., and von Wassermann, A., Handbuch der pathogenen mikroorganismen, Jena, 2nd edition, 1913, iii, 1016.
14. Penfold, W. J., *J. Hyg.*, 1911, xi, 487.
15. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 270.
16. Smith, T., *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 6*, 1894, 22.
17. Smith, T., and TenBroeck, C., *J. Med. Research*, 1914–15, xxxi, 503.
18. M'Gowan, J. P., and Wang, C. Y., *J. Path. and Bacteriol.*, 1915, xx, 21.
19. M'Gowan, J. P., Pathology and Epidemiology of Swine Fever, *Bull. Edinburgh and East of Scotland Agric. College*, July 1915.
20. M'Gowan, Pathology and Epidemiology of Chicken-Cholera, *Bull. Edinburgh and East of Scotland Agric. College*, July 1915.

THE EFFECTS OF EXPOSURE TO COLD UPON EXPERIMENTAL INFECTION OF THE RESPIRATORY TRACT.*

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Exposure to cold has long been considered an important factor in the incidence of many respiratory diseases. Even with our increasing knowledge of the predominating part of bacterial infection, the belief is still general among clinicians that chilling of the body exerts a predisposing influence of considerable importance.

In reviewing the general subject of the effect of atmospheric conditions upon bodily health and efficiency, the New York State Ventilation Commission was impressed with the comparative lack of accurate data concerning the physiological effect of low temperatures, the majority of recent observers having interested themselves mainly with the study of various degrees of heat.

Experimental studies of the influence of cold as well as of heat have therefore formed a part of the work of the Commission for the past 2 years. The experiments here reported constitute a portion of these studies.

EXPERIMENTAL.

The organism chosen for inoculation was *Bacillus bovisepticus*, or snuffles bacillus, which belongs to the hemorrhagic septicemia group and is pathogenic for the rabbit. It is a small, round bacillus with a marked tendency to bipolar staining, particularly in blood films made from animals dying from infection caused by it. The disease produced under natural conditions is generally a true septicemia, but in many cases is localized in the upper respiratory passages,

* Conducted under the auspices of the New York State Ventilation Commission.

when it is commonly known as snuffles. This condition is characterized by a typical discharge of yellowish mucus from the nostrils and may terminate in recovery or in a fatal pneumonia with or without general septicemia.

It will thus be seen that the conditions produced in the rabbit by this bacillus are similar to some of the respiratory infections in man, such as pneumonia and influenza. The organism was selected for use in our work because of this fact and because of the relative difficulties which have attended previous experiments with acute respiratory infections, particularly those caused by the pneumococcus.

Experiment 1.—This experiment was conducted during the late winter months of 1915 and 1916. Thirty-seven experimental rabbits were used in thirteen separate series of from two to four animals, each with an equal number of controls.

The experimental rabbits were kept in an incubator at warm temperatures for periods varying from 1 day to 1 week. In eleven series this temperature was between 85–90° F., and in two it varied from 70–76° F. and 63–78° F., respectively, being, however, at the higher level most of the time. The animals were then inoculated by spraying the nasal mucous membrane or the throat with a small amount of the live culture of *Bacillus bovisepticus*, emulsified in salt solution. After inoculation they were immediately chilled by exposing them to the outside weather for periods varying from ½ to 3 hours and at a temperature of 20–56° F. In the majority of the experiments the fur of the animals was wet with water at about body temperature, to facilitate chilling upon exposure. The details of temperature, time of exposure, method of inoculation, etc., are shown in the table under each experiment.

The control animals, also thirty-seven in number, were kept constantly at a temperature of 65–70° F. and were inoculated at the same time and in the same manner as the experimental animals of the corresponding series. None of these were wet.

Results.—Of the experimental rabbits five died of pneumonia, and one of general septicemia, while nine were made ill, but recovered. Of the controls three died and six others were infected, but recovered. In every case when death resulted, the animal was autopsied, the character of the lesions noted, and the specific organism isolated from the heart's blood.

It will thus be seen that of the experimental animals fifteen, or 40 per cent, reacted, and only nine, or 24 per cent, of the controls. It is also worthy of note that this difference in reaction between the experimental and the control groups was harmoniously distributed

throughout the entire thirteen series of experiments. In only two series (Nos. 11 and 12), did the number of reactions in the control group exceed that of the experimental animals. The details of this experiment are shown in Table I.

It seemed desirable to note the effect of reversing the conditions; that is, to determine the result of a change from a cold to a hot temperature. It was possible to conduct only two series in this experiment.

Experiment 2.—Four animals were kept at out of door temperature for 2 days, and after inoculation as in the previous experiment were placed in an incubator at 78–80°F. for from 6 to 12 days. A similar number of controls, inoculated in the same way, were kept constantly at room temperature (65–70° F.).

Results.—Of the experimental animals all reacted, of which one died. Of the controls only two reacted, one of which also died. A detailed description is given in Table II.

From the results obtained in the two foregoing experiments it seemed desirable to attempt one further experiment in which three modifications of the experimental conditions were considered together. (1) A change from outside temperature (59° F.) to incubator temperature (80° F.), (Series 16). (2) A change from room temperature (65–70° F.) to incubator temperature (80° F.), then down to a low temperature (50–60° F.), and from that back to room temperature (65–70° F.), (Series 17). (3) A modification of the conditions of Experiment 1; namely, chilling from a high temperature (80° F.) to a low one (57° F.), and then kept at 65-70° F. (Series 18).

Experiment 3.—All three series of this experiment were carried on simultaneously with two animals in each series, and with one set of two control animals which were kept continuously at room temperature (65–70° F.).

Results.—In Series 16, subjected to a change from low to high temperature, both animals died of pneumonia. In Series 17, subjected to several changes from room temperature, to high, then to low, and back to room temperature, both animals reacted, of which one died of pneumonia. In Series 18, subjected to a change from high to low temperature, only one animal reacted slightly with snuffles. Neither of the two controls reacted. Table III shows these results in detail.

TABLE I.

Series.	Heated and exposed.			Controls, kept at room temperature (65–70°F.).		
	No. of rabbits.	Reactions.		No. of rabbits.	Reactions.	
		Died.	Sick.		Died.	Sick.
1	4 24 hrs. at 87°F.; chilled to 45°F. for 1 hr. 2, fur wet; 2, fur dry. 2, nose; 2, throat.	2 Pneumonia. 1, fur wet; 1, fur dry. Death in 48 hrs.	1 General reaction; could hardly move; no appetite. Recovered.	4	0	1 General reaction. Recovered.
2	2 1 wk. at 85–89°F.; chilled to 40°F. for $1\frac{1}{4}$ hrs. Fur dry. 1, nose; 1, throat.	0	1 Snuffles. Recovered.	2	0	0
3	2 1 wk. at 85–89°F.; chilled to 38°F. for 3 hrs. Fur dry. 1, nose; 1, throat.	0	1 Snuffles. Recovered.	2	1 Pneumonia. Death in 1 wk.	0
4	4 1 wk. at 85–90°F.; chilled to 38°F. for $1\frac{1}{2}$ hrs. Fur dry. 2, nose; 2, throat.	0	0	4	0	0
5	4 1 wk. at 85–90°F.; chilled to 56°F. for 1 hr. Fur dry. 2, nose; 2, throat.	0	0	4	0	0
6	4 1 wk. at 85–90°F.; chilled to 20°F. for $\frac{1}{2}$ hr. Fur dry. 2, nose; 2, throat.	1 Snuffles; septicemia. Death in 10 days.	0	4	0	0

7	4 1 wk. at 85–89°F.; chilled to 45°F. for 2 hrs. Fur dry. 2, nose; 2, throat.	0	1 Snuffles. Recovered.	4	0	1 Snuffles. Recovered.
8	3 1 wk. at 85–90°F.; chilled to 40°F. Fur wet.	0	2 Snuffles. Recovered	3	0	0
9	2 3 days at 70–76°F.; chilled to 25°F. for ½ hr. Fur wet. 1, nose; 1, throat.	1 Pneumonia. Death in 2 days. Inoculated in throat.	1 Snuffles. Inoculated in nose. Recovered.	2	2	0
10	2 3 days at 85–93°F.; chilled to 52°F. for ½ hr. Fur wet. 1, nose; 1, throat.	1 Pneumonia. Death in 11 days. Inoculated in nose.	0	2	0	0
11	2 24 hrs. at 82–87°F.; chilled to 27°F. for ½ hr. Fur wet. 1, nose; 1, throat.	0	0	2	0	1 Snuffles. Recovered. Inoculated in nose.
12	2 24 hrs. at 85–90°F.; chilled to 31°F. for ½ hr. Fur wet. 1, nose; 1, throat.	0	1 Snuffles. Severe reaction. Recovered. Inoculated in nose.	2	0	2 Snuffles. Mild reaction. Recovered.
13	2 4 days at 63–78°F.; chilled to 32°F. for ⅓ hr. Fur wet. 1, nose; 1, throat.	1 Pneumonia. Death in 3 days. Inoculated in throat.	1 Snuffles. Recovered. Inoculated in nose.	2	0	1 Snuffles. Recovered. Inoculated in nose.
Total	37	6	9	37	3	6

TABLE II.

Series.	Kept cold in the open, then heated in incubator.			Controls, kept at room temperature (65–70°F.).		
	No. of rabbits.	Reactions.		No. of rabbits.	Reactions.	
		Died.	Sick.		Died.	Sick.
14	2 2 days at 41°F.; 12 days at 80°F. 1, nose; 1, throat.	0	2 Snuffles. 1, inoculated in nose, reacted in 4 days; 1, inoculated in throat, reacted in 6 days.	2	0	1 Reacted in 12 days; Inoculated in nose.
15	2 2 days at 35–40°F.; 6 days at 78°F.	1 Pneumonia. Inoculated in throat.	1 Snuffles. Inoculated in nose.	2	1 Pneumonia. Inoculated in throat.	0
Total	4	1	3	4	1	1

TABLE III.

Experimental.									Controls, kept at room temperature (65–70° F.).		
Series 16.			Series 17.			Series 18.					
48 hrs. at 59° F.; inoculated and kept at 80° F. 1, nose; 1, throat.			48 hrs. at 65–70° F.; inoculated; 24 hrs. at 80° F.; 4 days in open (50–60° F.); then at room temperature (65–70° F.).			2 days' incubation (80° F); inoculated; fur wet; 20 min. at 57° F.; then kept at 65–70° F.					
No. of rabbits.	Died.	Reacted.	No. of rabbits.	Died.	Reacted.	No. of rabbits.	Died.	Reacted.	No. of rabbits.	Died.	Reacted.
2	2 Pneumonia.	0	2	1 Pneumonia. Death in 6 days. Inoculated in nose.	1 Mild attack of snuffles in 7 days. Inoculated in throat.	2	0	1 Snuffles in 3 days. Inoculated in throat.	2	0	0

DISCUSSION.

Other experimental studies of respiratory disease have been numerous, but few of these deal directly with the associated problem of the effect of chill with which we are at present concerned.

Lipari (1) in 1888 reports the results of intratracheal injections of pneumonic sputum in guinea pigs and rabbits which, after exercise by running, were chilled either by cold baths at 3°C. or by the application of ether to the skin after the animals had been shaved. Of eight chilled animals six died, while only two of twelve controls were affected.

Lode (2) in 1897 shaved guinea pigs over one-half to two-thirds of the body, exposed them to heat (37°C.) for half an hour, and then chilled them in draughts at open windows. Using Buchner's apparatus he exposed them to the spray of cultures of Friedländer's bacillus. Of eleven chilled animals seven died, while only three of eleven controls succumbed. In a similar inhalation experiment with tubercle bacilli, of five chilled animals all died, but only two of four controls. He also used Friedländer's bacillus, cholera bacillus, and *Staphylococcus aureus* subcutaneously in his series, as well as anthrax with a few chickens and rats. Altogether, including both inhalation and subcutaneous methods, he reports that forty-six of fifty-four chilled animals (85 per cent) died, and only nine of forty-five controls (20 per cent).

Pasteur's (3) experiments upon chickens made susceptible to anthrax by partial immersion in cold water are well known, but these as well as others not dealing with direct respiratory infection need not be considered here.

Chodounský (4), who has covered the whole field of the relation between exposure to cold and disease perhaps more extensively than any writer, comes to the conclusion that such exposure plays no part in determining the incidence of any of the various so called cold diseases. As a part of his contention he offers considerable experimental evidence concerning respiratory infections.

The experimental animals in Chodounský's studies were chilled by exposure to ice baths and cold draughts until their rectal temperature was lowered from 1–7°C. He employed several kinds of animals and several different pathogenic bacteria.

Virulent pneumococcus cultures by inhalation were used with one rabbit and one guinea pig with similar animals for controls. The rabbit was infected and died, but the guinea pig as well as the two controls survived.

Intravenous injections of pneumococci were also employed with practically identical results in the chilled and control animals.

Virulent cultures of Friedländer's bacillus were used by intratracheal injections in dogs and by inhalation with Buchner's apparatus in guinea pigs. Six dogs and two guinea pigs with a similar number of controls were employed. Of the dogs two experimental animals and three controls were infected; of the guinea pigs one each of the experimental animals and controls.

Chodounský also used cultures of Friedländer's bacillus attenuated by heat to determine whether these cultures might infect chilled animals. Intratracheal injections in four experimental dogs and four controls failed to infect any. Later, one of the controls was injected with a virulent culture and promptly succumbed. Chodounský also experimented with intravenous injections of pneumococcus in guinea pigs, with intraperitoneal injections of Friedländer's bacillus in rats, and with subcutaneous injections of diphtheria bacilli either alone or in combination with streptococcus, and in all these found no difference in reaction between the chilled and control animals.

More interesting and perhaps even more instructive are the results of Chodounský's self-experimentation. He describes himself as a man 57 years of age, subject to catarrh, frequent bronchitis, and lumbago. He reports in detail the results of twenty-seven separate experiments upon the effect upon himself of severe exposure to cold. After hot baths, after ice cold baths, and even when suffering from an acute coryza, he exposed himself naked to cold air and draughts at temperatures from 3–12°C. for periods of time varying from 20 minutes to 1 hour. Such exposure often caused severe shaking chills, but no permanent effect of any kind and no symptoms of any of the pathological conditions usually ascribed to exposure to cold.

Recently Kline and Winternitz (5), in the course of their studies of experimental pneumonia in rabbits, found that in the case of animals which were ex-

posed to cold (4°C.), after the ingestion of alcohol every day for periods varying from 1 to 9 days, four out of seven died, two of which showed intense laryngitis, tracheitis, and bronchitis, and one a bronchopneumonia. One other animal was killed at the end of 3 days and showed patchy congestion in the lungs. In the two remaining animals cultures of pneumococcus were injected intratracheally and death occurred in both, with lesions in the lungs and upper air passages. Pneumonia rarely occurred after such injections in animals not exposed to the effects of cold and of alcohol.

The weight of the experimental evidence thus reported appears to be against Chodounský's contention that exposure to cold has no influence upon respiratory disease, and with this evidence the results of our experiments are in accord.

The method of action of these secondary factors in infection is open to discussion. The lowering of general or specific resistance to infection may be considered. It has been shown by a number of observers including ourselves (6) that the production of such antibodies as hemolysins and agglutinins is affected by atmospheric temperature, especially, however, by heat. On the other hand, changes in the local resistance of the mucous membranes of the upper air passages may be more important. That definite circulatory changes do occur in the upper air passages as a result of varying atmospheric temperature has been observed in animals and also in clinical experiments upon human subjects by one of us (7) in collaboration with Cocks, which corroborated similar observations by Hill and Muecke (8). In these experiments the results appeared to be due to the direct action of air upon the mucous membranes and not to a reflex from the surface of the body. The possible concern of vagus control in this action, as suggested by Kline and Winternitz (5), is worthy of serious consideration. The fact that infection of the normal respiratory tract is experimentally difficult tends to emphasize the probable importance of the local changes in the mucous membranes favoring the entrance of bacteria as the main predisposing factor in the problem under consideration.

CONCLUSIONS.

1. Respiratory infection of rabbits with *Bacillus bovisepticus* (snuffles) is favored by chilling the animals after they have been accustomed to heat.

2. The character of this disease, which occurs frequently in rabbits under natural conditions, makes the application of the experimental results to similar respiratory conditions in man less open to objection than in similar experiments with other infections.

3. The weight of experimental evidence, including our own, does not justify the elimination of exposure to cold as a possible though secondary factor in the incidence of acute respiratory disease.

4. From the limited data of our last two experiments it is suggested that any marked change of temperature predisposes rabbits to this infection, the severity of which varies with the amount of change, and that a change from low to high temperature has an even more marked effect than that from high to low.

We wish to acknowledge our obligation to Mr. I. Weinstein for valuable assistance in conducting the details of the animal work in some of the experiments.

BIBLIOGRAPHY.

1. Lipari, G., *Morgagni*, 1888, xxx, 523, 575, 651.
2. Lode, A., *Arch. Hyg.*, 1897, xxviii, 344.
3. Vallery-Radot, R., The Life ot Pasteur, New York, 1902, ii, 61.
4. Chodounský, K., Erkältung und Erkältungskrankheiten, Vienna, 1907.
5. Kline, B. S., and Winternitz, M. C., *J. Exp. Med.*, 1915, xxi, 304.
6. Winslow, C.-E., Miller, J. A., and Noble, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 93.
7. Miller, J. A., and Cocks, G. H., *Tr. Am. Climat. and Clin. Assn.*, 1915, xxxi, 31.
8. Hill, L., and Muecke, F. F., *Lancet*, 1913, i, 1291.

STUDIES ON HETEROGENEOUS HEMOLYTIC SERA.

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In 1911 Forssman (1) refuted the theory of the specificity of antibodies which had been generally admitted since the discovery of bacteriological antibodies by Pfeiffer and of hemolysins by Bordet. Forssman demonstrated that rabbits immunized with organs of the guinea pig produced a hemolysin against sheep red blood corpuscles. The hemolytic serum thus obtained possessed greater hemolytic power for sheep red corpuscles than for those of the guinea pig. The minimum hemolytic dose for sheep red corpuscles was 0.001 cc., and for those of the guinea pig, 0.03 cc. It was afterwards found that the antigen-producing sheep hemolysin was found in other animals as well as in the guinea pig, *i.e.*, horse, cat, dog, mouse, hen, pigeon, turtle, toad (*Bufo*), carp, tench, pike, and eel, and in the cells of some bacteria (*Bacillus paratyphosus B* and Gärtner's bacillus). Forssman's finding that the antigen-producing heterogeneous sheep hemolysin is present in the organs of animals has been confirmed by Orudschiew (2) in serum, and by Doerr and Pick (3) in urine, into which it passes from the cells of the organs. According to Doerr and Pick (4), it is not found in the red corpuscles of these animals. Orudschiew, who first studied the problem, came to this conclusion because after immunizing rabbits with guinea pig red corpuscles he obtained serum containing hemolysin for guinea pig red corpuscles but not for sheep corpuscles. Doerr and Pick (5) state that an inverse relation exists, as a rule, between the organs of the animal and the red corpuscles. When the organs contain the antigen, the red corpuscles do not (horse, guinea pig, dog, mouse[1]), and *vice versa* (sheep). Morgenroth (6) held the same view. Friedberger and Schiff (7) state that one of three rabbits immunized with human red corpuscles produced a serum that dissolved sheep red corpuscles in a dose of 0.01 cc.;[2] but apparently Friedberger did not attach much value to this one positive experiment, because in his later works (8, 9) human red corpuscles are not mentioned as containing heterogeneous antigens.

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[1] In another work Doerr and Pick (4) added the hen and the turtle to this list.

[2] The other two rabbits produced a serum of which the minimum dose did not exceed that of a normal animal; moreover, the authors do not cite protocols, and one cannot see whether the hemolysis in the only positive experiment (at 0.01 cc.) was complete or partial.

My experiments show that the connective tissue of animals (fat of guinea pigs) possesses an antigen-producing hemolysin against sheep red corpuscles. This fact, in connection with the works of Doerr and Pick (5) demonstrating that the heterogeneous sheep antigen found in animal organs was a nucleoprotein, made me believe that the cells not deprived of a nucleus, for example the red corpuscles with a nucleus derived from the same mesoderm as the connective tissue, should also contain heterogeneous sheep antigen when the organs of the same animal possess it. The experiments confirmed this hypothesis. The experiments were made with red corpuscles of the hen, whose organs, as shown by Doerr and Pick, contain a heterogeneous antigen-producing hemolysin against sheep red corpuscles.

EXPERIMENTAL.

Hen red corpuscles were washed three times in physiological salt solution. To the sediment left after centrifugation was added an equal part of salt solution, and the mixture was injected into rabbits. The serum was inactivated at 56° C. for ½ hour. Its hemolytic power was titrated in the presence of 0.1 cc. of guinea pig complement, which by itself is unable to cause hemolysis, and of 1 cc. of a 5 per cent suspension of the sediment of the sheep red blood corpuscles, in a total volume of 3 cc. The tubes were kept at 37° C., and the result of the experiment was read after 1 hour.

Rabbit 1.—Weight 1,625 gm. Jan. 8, 1.5 cc.[3] of hen red corpuscles injected into the ear vein. Jan. 15, 1.2 cc. injected. The hemolytic power of the serum tested on Jan. 22 and 26 was identical. 0.01 cc. produced complete hemolysis of sheep corpuscles; 0.005 cc., almost complete hemolysis.

Rabbit 2.—Weight 1,285 gm. Jan. 24, 2.0 cc. of hen red corpuscles injected into the ear vein. Jan. 31, 5 cc. injected into the abdomen. Hemolytic power: Feb. 8, 0.001 cc., complete hemolysis of sheep corpuscles; 0.0008 cc., almost complete hemolysis.

Rabbit 3.—Weight 1,775 gm. Jan. 24, 3.0 cc. of hen red corpuscles injected into the ear vein. Jan. 31, 2.5 cc. injected in two doses, first 0.25 and after ½ hour, 2.25 cc. Hemolytic power: Feb. 8, 0.002 cc., complete hemolysis of sheep corpuscles; 0.0016 cc., incomplete hemolysis.

Rabbit 4.—Weight 1,370 gm. Jan. 24, 2.0 cc. of hen red corpuscles injected into the ear vein. Jan. 31, 2.5 cc., in two doses. Hemolytic power: Feb. 8, 0.00125 cc., complete hemolysis of sheep corpuscles; 0.0011 cc., incomplete hemolysis.

Rabbit 5.—Weight 1,220 gm. Feb. 24, 2.5 cc. of hen red corpuscles injected into the ear vein. Mar. 2, 2.0 cc., in two doses. Hemolytic power: Mar. 10, 0.001 cc., complete hemolysis of sheep corpuscles; 0.0008 cc., incomplete hemolysis.

[3] Quantity of sediment.

Rabbit 6.—Weight 1,155 gm. Feb. 24, 2.5 cc. of hen red corpuscles injected intravenously. Mar. 2, 2.0 cc., in two doses. Hemolytic power: Mar. 10, 0.0025 cc., complete hemolysis of sheep corpuscles; 0.002 cc., almost complete hemolysis.

Rabbit 7.—Weight 1,510 gm. Feb. 24, 1.5 cc. of hen red corpuscles injected intravenously. Mar. 2, 2.5 cc. Hemolytic power: Mar. 10, 0.00125 cc., complete hemolysis of sheep corpuscles; 0.0011 cc., almost complete hemolysis.

TABLE I.

No. of rabbit.	1	2	3	4	5	6	7
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Hemolytic power of serum............	0.01	0.001	0.002	0.00125	0.001	0.0025	0.00125

In Table I are indicated the minimal doses of serum of each rabbit that gave complete hemolysis of sheep red corpuscles.

The protocols show that hen red corpuscles contain the same heterogeneous antigen-producing hemolysin against sheep red corpuscles as the organs of this animal. At any rate the quantity of heterogeneous antigen in the red corpuscles of the hen is not less than that in its organs, and the heterogeneous sera obtained possess a high hemolytic power. I believe that these experiments invalidate the law of Doerr and Pick.

The organs as well as the red corpuscles of the same animal may contain heterogeneous antigen; if it is not found, however, in the red corpuscles of mammals whose organs possess it, this may be attributed to the absence of a nucleus containing a large amount of nucleoprotein of cellular body. It is possible that the heterogeneous antigen may be found also in the red corpuscles of the pigeon, turtle, and toad. According to Amako (10), the organs of these animals also contain heterogeneous antigens.

The paradoxical nature of heterogeneous hemolytic sera is evident from these experiments. This refers to the fact that the heterogeneous sera possessing a high dissolving action on sheep red corpuscles contain a very small quantity of hemolysin against the red corpuscles of the species whose organs were used as antigen.

1 cc. of the hemolytic sera used contained about 1,000 hemolytic units against sheep red corpuscles (Table I), but they were almost

inactive against hen red corpuscles from which they were produced[4] (Table II).

TABLE II.

No. of rabbit.	0.1 cc. of serum.	0.02 cc. of serum.	0.01 cc. of serum.
2	Tr.*	Tr.	Tr.
3	I.	"	"
4	Tr.	"	"
5	"	"	"
6	"	"	"
7	"	"	"

*In the tables Tr. indicates trace of hemolysis; I., incomplete hemolysis; Sl., slight hemolysis; N., no hemolysis; C., complete hemolysis.

The experiment was made as follows: 3 cc. of liquid in each tube, in the presence of 0.1 cc. of guinea pig complement (which alone is unable to hemolyze hen red corpuscles) and of 1 cc. of 5 per cent suspension of hen red corpuscles washed three times.

The almost complete absence of hemolysins in the red corpuscles of the hen which had furnished the antigen, and the formation of hemolysin against the red corpuscles of an animal of another species (sheep) disprove more forcibly the theory of the specificity of antibodies than did the phenomenon of the simultaneous presence of the specific antigen and an accidental one (common to different animals and bacteria) inside the cell.

[4] A number of sera from normal rabbits were treated. Their power of dissolving hen red corpuscles was as follows:

Normal rabbits.	0.1 cc.	0.3 cc.	0.5 cc.	0.7 cc.	1.0 cc.
1	I.	I.	I.	Almost C.	Almost C.
2	"	"	"	I.	I.
3	Tr.	Tr.	Tr.	Tr.	"
4	I.	I.	I.	I.	Almost C.
5	"	"	"	"	I.

Therefore, 0.1 cc. given to four normal rabbits produced even a stronger hemolysis than when given to immunized animals. Yet 0.02 and 0.01 cc. never showed any trace of hemolysis. This fact did not allow me to disprove the presence of immune hemolysins against hen red corpuscles.

These facts were discovered by Forssman, but neither he nor the other workers who studied the problem of heterogeneous hemolysins insisted upon it sufficiently. Forssman noticed that his sera produced by rabbits which had been immunized with guinea pig organs contained in 1 cc. 200 and 1,000 hemolytic units against sheep red corpuscles, and only 25 to 30 units against guinea pig red corpuscles; but he stated that the hemolysin against sheep red corpuscles was the principal one, and that the other, against guinea pig red corpuscles, was accessory. In my experiments the accessory hemolysin has not been definitely discovered, because even 0.1 cc. was unable to hemolyze completely one unit of hen red corpuscles (Table II).

Thus the fact established by Forssman becomes particularly salient in connection with the heterogeneous sera that I obtained. Moreover, the use of animal organs for immunization may suggest the objection that the absence or slight formation of homologous hemolysins was due to the organ specificity of the antigen. With red corpuscles the question of organ specificity is excluded.

The question therefore arises: What if the antigenic action of hen red corpuscles were only an exception? Could we not explain the absence of specific hemolytic antigen in hen red corpuscles by their having lost all receptors of their species, such as, for instance, the crystalline of adult animals? The following experiments contradict this hypothesis. Hen red corpuscles derived from specific hemolytic antigen possess specific agglutinogens. After immunization of rabbits a great quantity of agglutinin to hen red corpuscles was formed in the serum (Table III).

TABLE III.

No. of rabbit.	0.01 cc. of serum.	0.002 cc. of serum.	0.001 cc. of serum.	0.0002 cc. of serum.	0.0001 cc. of serum.	0.00005 cc. of serum.	0.00002 cc. of serum.	0.0000125 cc. of serum.	0.00001 cc. of serum.	0.000005 cc. of serum.
2	+	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	Sl. +	−			
4	+	+	+	+	+	Sl. +	−			
5	+	+	+	+	+	+	+	+	+	−
6	+	+	+	+	+	+	+	+	+	−
7	+	+	+	+	+	+	+	+	+	−

0.2 cc. of a 5 per cent suspension of hen red corpuscles was added to 1 cc. of serum dilution; the tubes were kept at 37° C. for 2 hours and then on ice until morning.

The sera in question contain not only heterogeneous hemolysins but also heterogeneous agglutinins (Table IV).

TABLE IV.

No. of rabbit.	0.1 cc. of serum.	0.02 cc. of serum.	0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.	0.00125 cc. of serum.	0.001 cc. of serum.
2	+	+	+	+	+	Sl. +	−
3	+	+	+	+	−		
4	+	+	+	+	+	Sl. +	−
5	+	+	+	+	+	−	
6	+	+	+	+	Sl. +	−	
7	+	+	+	+	−		

The experiment with sheep red corpuscles was carried out like that of Table III.

Forssman and Hintze (11) several times found small quantities[5] of agglutinin to sheep red corpuscles in sera of rabbits immunized with guinea pig organs. Doerr and Pick (4) state that most of the heterogeneous sera that they obtained from rabbits immunized with horse or guinea pig organs were devoid of hemagglutinins; in another place (5) they state that all the sera produced by rabbits immunized with horse organs were devoid of agglutinins to sheep red corpuscles. Considering, however, that heterogeneous sera of rabbits immunized with hen red corpuscles always possess heterogeneous hemagglutinins, one must reject the statement of Doerr and Pick (4) who thought that the absence of agglutinins in heterogeneous sera was one of the characteristics distinguishing them from homologous sera. The difference in the results of Forssman's experiments and mine on the one hand, and Doerr and Pick's on the other, may be explained by the fact that some heterogeneous sera (immunized with horse organs) are totally devoid of hemagglutinins, some possess them accidentally (immunized with guinea pig organs), and some, such as the sera that are the special objects of this investigation, always contain agglutinins to sheep red corpuscles. The latter sera, produced by rabbits immunized with hen red corpuscles, possess less heterogeneous agglutinins, however, than heterogeneous hemolysins (Tables I and IV).

[5] The quantity is not exact.

Heterogeneous sera of rabbits, immunized with hen red corpuscles, do not contain a hemolysin against the red corpuscles of the ox, like the heterogeneous sera studied by earlier authors (Table V).

TABLE V.

No. of rabbit.	0.1 cc. of serum.	0.02 cc. of serum.	0.01 cc. of serum
2 3 4 5 6 7	No hemolysis.	No hemolysis.	No hemolysis.

The experiment with red corpuscles was carried out like that of Table I.

Like the heterogeneous sera studied earlier, the hemolytic sera of rabbits immunized with hen red corpuscles are toxic to guinea pigs and kill them, with the clinical picture of anaphylactic shock.

Serum 2.—A guinea pig weighing 265 gm. was injected with 1 cc. of serum, into the jugular vein. Death followed in 5 minutes. 30 seconds after the beginning of the experiment the guinea pig appeared to have a chill; convulsions followed and the animal fell on its side; in this position were observed tonic and clonic convulsions of the extremities, shortness of breath, and loss of reflexes. A foaming serous liquid, not tinged with blood, came from the mouth and nostrils. Autopsy: thrombus in heart cavity and vessels, and in the vena cava inferior; the lungs were inflated, covering a large part of the heart; no lung hemorrhage; the lungs on section showed a foaming serous liquid.

A guinea pig weighing 170 gm. received 0.5 cc. of serum into the jugular vein. Death followed in 6.5 minutes. There was the same clinical and anatomical picture as in the preceding case.

A guinea pig weighing 170 gm. received 0.3 cc. of serum into the jugular vein. Death followed after 5.5 minutes. The same clinical and anatomical picture.

A guinea pig weighing 170 gm. received 0.1 cc. of serum into the jugular vein The only effect observed was a brief chill.

Serum 3.—A guinea pig weighing 185 gm. received 1 cc. of serum into the jugular vein. Death followed after 7 minutes. Clinical picture as in the preceding cases. Autopsy: thrombus in the right heart and vessels; lungs inflated, edematous, slight hemorrhage.

A guinea pig weighing 150 gm. received 0.3 cc. of serum into the jugular vein. The only effect was a brief chill.

Serum 4.—A guinea pig weighing 260 gm. received 1 cc. of serum into the jugular vein. Death after 3.5 minutes. Clinical picture as in the preceding cases but without foaming liquid. The same anatomical changes as in the animals killed by Serum 2, but the lung inflation was insignificant and there was no edema.

A guinea pig weighing 175 gm. received 0.5 cc. of serum into the jugular vein. Death followed after 5 minutes. The same clinical picture as in the animals killed by Serum 2. Autopsy: thrombus only in the right heart and vessels; lungs slightly inflated, edematous.

A guinea pig weighing 170 gm. received 0.3 cc. of serum into the jugular vein, with no effect.

Heterogeneous hemolysins of rabbits immunized with hen red corpuscles can be bound with their antigen (Table VI).

TABLE VI.

Date.	No. of serum.	0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.	Experiment of binding hen red corpuscles with homologous serum No. 8.		
					0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.
Mar. 5	2*	N.	N.	N.	C.	I.	Tr.
	4*	"	"	"			
	5†	I.	Tr.	Tr.			
" 10	6†	Tr.	"	"	"	Almost C.	I.
	7†	"	"	"			

* A suspension was used of 3 cc. of hen red corpuscle sediment in 10 cc. of physiological salt solution.

† A suspension was used of 3 cc. of hen red corpuscle sediment in 20 cc. of physiological salt solution.

The experiment was carried out as follows: A suspension of hen red corpuscles was freed of serum by washing and was mixed with equal parts of heterogeneous hemolysin prepared in the proportion of 1:50; the mixture was kept at 37°C. for 1 hour, then centrifuged. The hemolytic power of the liquid against hen red corpuscles was tested at 37° C. during 2 hours.

The kidneys of the hen, which according to Doerr and Pick contain heterogeneous antigens, also bind the hemolysins in question, but less completely (Table VII).

TABLE VII.

Date.	No. of rabbit.	0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.	Experiment of binding suspension of hen kidney with homologous serum No. 8.		
					0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.
Mar. 12	5	C.	Tr.	Tr.	C.	Almost C.	I.
	6	I.	"	"			
	7	"	"	"			

The experiments were carried out in the same way as those of Table VI. The kidney suspension was prepared in the proportion of 3 gm. to 20 cc. of physiological salt solution.

The antigen found in guinea pig organs and producing heterogeneous hemolysin against sheep red corpuscles reveals inconstant relations in binding hemolysins produced by rabbits immunized with hen red corpuscles (Table VIII).

TABLE VIII.

Date.	No. of rabbit.	0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.	Experiment of binding suspension of guinea pig kidney with homologous serum No. 8.		
					0.01 cc. of serum.	0.003 cc. of serum.	0.002 cc. of serum.
Mar. 5	2	C.	C.	I.	C.	I.	Tr.
	4	"	I.	Tr.			
" 10	5	I.	Tr.	"	"	Almost C.	I.
	6	Tr.	"	"			
	7	"	"	"			

The experiments were carried out in the same way as those of Table VII.

The experiments given in Table VIII show that the heterogeneous antigen of guinea pig kidneys sometimes can be bound with the hemolysin in question in the same degree as the antigen of hen red corpuscles (Nos. 5, 6, and 7); in other cases the binding is very weak (No. 4) or is entirely absent (No. 2).

The reason for this inconstant binding of the heterogeneous hemolysin in question with a suspension of guinea pig kidneys is not clear.

The circumstance that the antigen used was not the same one which had generated the hemolysin may be of some importance. Further, it is possible that we have to deal with a phenomenon noticed by Bail and Margulies (12), who state that in 40 per cent of the experiments guinea pig organs do not bind the hemolysin at all or do so weakly. My experiments in binding the homologous hemolysin No. 8 (the right hand columns of Tables VI, VII, and VIII) with different heterogeneous antigens agreed with the observations of previous authors because the binding between that homologous hemolysin and the antigens of hen red corpuscles, and hen and guinea pig kidneys was not significant.

The experiments in Table IX were undertaken to ascertain the respective part of absorption and specific binding of the antibodies contained in the sera used.

TABLE IX.

No. of serum.	Sera after incubation with hen red corpuscles.			Sera after incubation with suspension of guinea pig kidney.					
	0.01 cc. of serum.	0.002 cc. of serum.	0.001 cc. of serum.	0.01 cc of serum.	0.002 cc. of serum.	0.001 cc. of serum.	0.0002 cc. of serum.	0.0001 cc. of serum.	0.00005 cc. of serum.
2	+	Sl. +	−	+	+	+	+	+	Sl. +
4	+	Sl. +	−	+	+	+	Sl. +	−	−
5	+	−	−	+	+	+	+	+	Sl. +
6	Sl. +	−	−	+	+	+	+	+	−
7	+	−	−	+	+	+	+	+	−

The sera used were prepared in the same way as in the experiments of Tables VI and VII. 1 cc. of the serum dilution was mixed with 0.2 cc. of a 5 per cent suspension of hen red corpuscles; the tubes were shaken and kept at 37° C. for 2 hours, then on ice until morning.

In this way was determined the quantity of hemagglutinins remaining after treating the serum (1) with hen red corpuscles possessing both heterogeneous antigen (hemolysin against sheep red cells) and homologous specific antigen (hemagglutinogen), and (2) with a suspension of guinea pig kidneys containing only heterogeneous antigen, producing a hemolysin against sheep red corpuscles.

From the quantity of hen hemagglutinins left, we are able to determine the relative importance of absorption (treating with guinea pig

kidneys deprived of hen agglutinogen) and of specific elective binding between antigen and antibody (treating with suspension of hen red corpuscles).

From Table III and the right hand columns of Table IX the intensity of the phenomenon of absorption is seen, as it has the power to deprive the serum of nine-tenths of its agglutinins. From Table III and the left hand columns of Table IX one can infer that the elective affinity between antigen and antibody is very great; it leaves in the serum only 0.001 (Nos. 2, 5, and 7), 0.002 (No. 6), and at the most 0.005 (No. 4) of its antibodies. Therefore, comparing the right and left hand columns of Table IX, we must assign the most important part to the specific elective affinity between antigen and antibodies; however, we should not lose sight of the part that non-specific absorption plays in the presence of a suspension of animal organs.

It is evident that heterogeneous hemolysins have been obtained by means of non-toxic antigen, such as hen red corpuscles injected into the veins of rabbits. This refutes the theory of Friedberger and Schiff who attributed the antigenic value of a suspension of organs to its toxic character.

The following experiments prove that the connective tissue of animals (guinea pigs) contains heterogeneous sheep antigen. The fatty tissue enveloping the intestines and kidneys of an exsanguinated guinea pig was rubbed and washed three times with physiological salt solution to remove every trace of serum. In this condition the adipose tissue becomes a strictly connective tissue deprived of any other textile element of the organism, except the ñerve ramifications.

Rabbit A.—Weight 1,590 gm. Oct. 26, 0.3 gm. of fat injected into the vein and 1 gm. into the abdomen. Nov. 5, 1 gm. of fat injected into the abdomen. Hemolytic power of the serum against sheep red corpuscles: Nov. 5, 0.01 cc., complete hemolysis; 0.003 cc., incomplete hemolysis; Dec. 13, 0.01 cc., complete hemolysis; 0.0025 cc., incomplete hemolysis.

Rabbit B.—Weight 1,260 gm. Jan. 8, 0.2 gm. of fat injected into the vein and 0.8 gm. into the abdomen. Jan. 17, 1.5 gm. of fat injected into the abdomen. Hemolytic power: 0.01, 0.003, and 0.0025 cc., complete hemolysis of sheep corpuscles; 0.002 cc., almost complete hemolysis.

Bail and Margulies supposed that the connective tissue possessed heterogeneous hemolytic antigen but quote no experiments to prove

it. The experiments described above fully demonstrate that the connective tissue possesses heterogeneous hemolytic antigen.

Bail and Margulies not only thought that the connective tissue possessed heterogeneous antigen; they supposed that the faculty of producing heterogeneous hemolysins, proper to organs of animals, was entirely due to the contents of heterogeneous antigen in the stroma of connective tissue. I undertook some experiments hoping to solve the problem by producing degeneration[6] of the parenchymatous cells of the organ (kidney). Rabbits were immunized with degenerated guinea pig organs, as it was thought that a strong diminution in the production of heterogeneous hemolysin would disprove the hypothesis of Bail and Margulies, because it would prove the antigenic value of the epithelial cells of the organ, which were excluded by degeneration. Unfortunately, the method failed. First, the animals withstood the immunization badly, and most of them died. The decrease, therefore, in the production of antibodies may have been due to cachexia of the rabbits rather than to any loss of antigen in the parenchymal cells injected. Besides, the histological picture of the kidneys after such injections was a very inconstant one; degeneration or necrosis was present in the convoluted tubules only, not in the straight ones; all the epithelial elements of the kidney became degenerated, and only the stroma and the glomeruli were morphologically unaltered. It is therefore impossible to draw any conclusion.

As they believe that only the connective tissue possessed heterogeneous antigen, Bail and Margulies undertook to prove that this antigen should be found in the organs of even those animals which were supposed not to have it, because the connective tissue, being a slightly differentiated formation, possesses identical characteristics in every animal. If, nevertheless, many animals never reveal heterogeneous antigen, that may be explained by the serum coming into contact with the heterogeneous antigen and binding it. Bail and Margulies believe that they succeeded in proving this hypothesis by their experiments. They bound heterogeneous antigens in organs of animals which possess them, by sera of animals which do not. But the results of the method they adopted are inconstant, as is evident from

[6] 1 cc. of saturated sublimate solution was injected subcutaneously.

the tables and protocols of their article. It therefore seemed necessary to choose a more certain way, such as the immunization of an animal with a mixture of an organ containing heterogeneous antigen with the serum of an animal whose organs do not possess heterogeneous antigen.

The procedure was as follows: Guinea pig kidneys were ground fine in physiological salt solution, 10 gm. of organ to 14 cc. of solution. The suspension was filtered through muslin and was mixed with rabbit serum, 2 cc. of suspension to 4 cc. of serum. The mixture was kept at 37°C. for 2 hours and was then injected into rabbits.

Rabbit A.—Weight 1,350 gm. Oct. 13, 3 cc. injected intravenously. Oct. 20, 5 cc. into the abdomen. Hemolytic power: Oct. 20, 0.006 cc., complete hemolysis of sheep corpuscles; 0.005 cc., incomplete hemolysis; Oct. 29, 0.002 cc., complete hemolysis; 0.0016 cc., incomplete hemolysis.

Rabbit B.—Weight 1,860 gm. Oct. 26, 10 cc. injected into the abdomen. Hemolytic power: Nov. 5, 0.003 cc., complete hemolysis of sheep corpuscles; 0.0025 cc., traces of hemolysis.

These experiments prove that a suspension of guinea pig organs conserves the faculty of producing hemolysins after a contact of 2 hours with the serum of a rabbit whose organs are deprived of heterogeneous antigen. In consequence, the serum is unable to deprive the organs of animals of their hemolytic antigen by its contact, and the absence of heterogeneous antigen in some species of animals cannot be explained by the neutralizing power of its serum.

SUMMARY.

Red corpuscles with nuclei contain heterogeneous sheep antigen when the organs of the same animal possess it.

The sera in question contain not only heterogeneous hemolysins but also heterogeneous agglutinins.

The hemolytic sera of rabbits immunized with hen red corpuscles are toxic to guinea pigs and kill them, the clinical picture being that of anaphylactic shock.

Heterogeneous hemolysins are obtained by means of non-toxic antigen (hen red corpuscles injected into veins of rabbits). This fact refutes the theory of Friedberger, who attributed the antigenic power of a suspension of organs to its toxic character.

BIBLIOGRAPHY.

1. Forssman, J., *Biochem. Z.*, 1911, xxxvii, 78.
2. Orudschiew, D., *Z. Immunitätsforsch., Orig.*, 1912–13, xvi, 268.
3. Doerr, R., and Pick, R., *Biochem. Z.*, 1913, l, 129.
4. Doerr and Pick, *Z. Immunitätsforsch., Orig.*, 1913, xix, 251, 612.
5. Doerr and Pick, *Biochem. Z.*, 1914, lx, 257.
6. Morgenroth, J., *Berl. klin. Woch.*, 1913, l, 561.
7. Friedberger, E., and Schiff, F., *Berl. klin. Woch.*, 1913, l, 1557.
8. Friedberger and Schiff, *Berl. klin. Woch.*, 1913, l, 2328.
9. Friedberger, E., and Goretti, G., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 91.
10. Amako, T., *Z. Chemotherap.*, 1912–13, i, 224.
11. Forssman, J., and Hintze, A., *Biochem. Z.*, 1912, xliv, 336.
12. Bail, O., and Margulies, A., *Z. Immunitätsforsch., Orig.*, 1913, xix, 185.

THE EFFECTS OF SERUM TREATED WITH AGAR.

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INTRODUCTION.

Bordet,[1] in 1913, showed that by adding to normal guinea pig serum a small quantity of a semifluid suspension of agar in physiological solution of sodium chloride a liquid was obtained, which, on intravenous injection into guinea pigs, caused typical anaphylactic intoxication. The mixture of serum and agar was kept at 37°C. for 2 hours, and then subjected to rapid centrifugalization to separate out the grayish sediment of the agar. The symptoms produced in the animals were tremors, dyspnea, micturition, and death after a few minutes. The autopsy which was performed immediately showed dilatation of the lungs with hemorrhagic areas varying in size and number, persistence of the heart beat, and a marked retardation of the coagulation of the blood. This picture is identical with that of anaphylaxis caused by the injection of antigen into a sensitized animal. Serum treated with agar acts in the same manner as anaphylatoxin (Friedberger). Of course, the intravenous injection of physiological salt solution kept for 2 hours at 37°C. in the presence of the suspension of agar, and freed from the sediment by centrifugalization, is innocuous to the guinea pig.

If agar is added to serum previously heated to 56°C., and then treated in the manner described above, the serum will be as harmless as the normal control serum not kept for 2 hours at a temperature of 37°C. in contact with the diluted agar.

Nathan[2] has shown that it is sufficient to add to 5 cc. of normal guinea pig serum 0.1 cc. of a suspension of 0.5 per cent agar, that is, 0.5 mg. of agar, to make this serum toxic after a minimum period of 1 to 1¼ hours at 37°C. These

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[1] Bordet, J., Le mécanisme de l'anaphylaxie, *Compt. rend. Soc. biol.*, 1913, lxxiv, 225; Gélose et anaphylatoxine, *ibid.*, 1913, lxxiv, 877; Qu'est-ce que l'anaphylaxie? *Ann. et Bull. Soc. roy. sc. méd. et nat. Bruxelles*, 1913, lxxi, 25.

[2] Nathan, E., Ueber Anaphylatoxinbildung durch Agar, *Z. Immunitätsforsch.*, *Orig.*, 1913, xvii, 478.

facts have been confirmed by Friedberger,[3] Haren,[4] Kraus and Kirschbaum,[5] Loewit and Bayer,[6] Tchernoroutzky,[7] and others.

For agar, a product which always contains relatively large quantities of nitrogen, pararabin may be substituted; that is, a part of the carbohydrates of agar. This pararabin of agar is practically free from nitrogen, containing only 0.23 per cent at the most. Guinea pig serum kept 2 or 3 hours at 37°C. in contact with this pararabin, on intravenous injection into the guinea pig, causes the same syndrome as serum treated with agar. Previous heating to 56°C. for 20 to 30 minutes prevents the serum from becoming toxic in the presence of the pararabin.[8]

Similar results are obtained by treating the serum with starch,[9] a suspension of inulin,[10] or a sodium compound of pectin free from nitrogen.[11] On the other hand, a solution of inulin does not give to the serum the properties of anaphylatoxin. The physical constitution of the product with which the serum is kept in contact at 37°C. for an adequate period seems to be of importance. For the moment, however, we shall leave this point.

Notwithstanding the marked similarity of symptoms observed on the one hand by reinjecting heterologous serum into a guinea pig sensitized 2 to 3 weeks before by this serum, and on the other hand by introducing guinea pig serum treated with agar or pararabin into the jugular vein of a normal guinea pig, the identity of the two phenomena is not fully established.

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[3] Friedberger, E., Handelt es sich bei der Anaphylatoxinbildung aus Agar-Agar nach Bordet um eine physikalische Absorptionswirkung, *Z. Immunitätsforsch., Orig.*, 1913, xviii, 323.

[4] Haren, P., Ueber die Giftigkeit arteigenen Serums und die Anaphylatoxinbildung aus Agar und Gelatine, *Z. Immunitätsforsch., Orig.*, 1913–14, xx, 673.

[5] Kraus, R., and Kirschbaum, P., Zur Frage der anaphylaktischen Vergiftung, *Wein. klin. Woch.*, 1913, xxvi, 783.

[6] Loewit, M., and Bayer, G., Die Abspaltung von Anaphylatoxin aus Agar nach Bordet, *Arch. exp. Path. u. Pharm.*, 1913, lxxiv, 478.

[7] Tchernoroutzky, Sur l'anaphylatoxine de Bordet, *Compt. rend. Soc. biol.*, 1913, lxxiv, 1213.

[8] Bordet, J., and Zunz, E., Production d'anaphylatoxine dans le sérum traité par de l'agar épuré de son azote (pararabine), *Z. Immunitätsforsch., Orig.*, 1914–15, xxiii, 42.

[9] Nathan, Ueber Anaphylatoxinbildung durch Stärke, *Z. Immunitätsforsch., Orig.*, 1913, xviii, 636.

[10] Nathan, Ueber Anaphylatoxinbildung durch Inulin, *Z. Immunitätsforsch., Orig.*, 1914–15, xxiii, 204.

[11] Kopaczewski, W., and Mutermilch, S., Sur l'origine des anaphylatoxines, *Compt. rend. Soc. biol.*, 1914, lxxvi, 782; *Z. Immunitätsforsch., Orig.*, 1914, xxii, 539.

Other analogies between these two groups of phenomena have been noted. A few days after the first parenteral injection of heterologous serum there appears, in the plasma of the animals submitted to the treatment, a proteolytic power for the sensitizing proteins.[12] More exactly, amino nitrogen is set free if the plasma of the sensitized animal is kept for 2 hours at 38°C. in the presence of the sensitizing serum. This amino nitrogen arises from the division of the peptic radicals, CONH, which constitute the essential part of the protein molecules and their immediate derivatives, proteoses, peptones, and peptides. This special proteolytic power acquires its maximum of intensity at the time when the sensitized animal reaches the anaphylactic state; that is, at the period during which the reinjection of the sensitizing serum produces symptoms of anaphylactic shock. This period, for example, in dogs after a sensitizing intravenous injection of ox serum is 15 days.[13]

If guinea pig serum is kept for 2 or 3 hours at 38°C. in the presence of agar or pararabin, disintegration of the proteins takes place as in the preceding case. This phenomenon does not occur[14] when the serum has been kept 20 or 30 minutes at 56°C. In this case also, a remarkable agreement is observed between the results obtained with animals sensitized by serum in a state of anaphylaxis, and the results obtained after treatment of normal guinea pig serum with agar or pararabin.

The importance of these experiments for the comprehension of anaphylaxis and its bearing on immunity will be readily conceded, although it would be premature to make any assertions on this subject. The study of the effects of serum treated with agar must first be extended to other species of animals. In collaboration with Bordet, one of us undertook these tests with rabbits and dogs, but the investigations were interrupted before their completion. Nevertheless, the results of the first experiments with rabbits tend to confirm the observations made with guinea pigs, considering the differ-

[12] Abderhalden, E., Abwehrfermente, Berlin, 4th edition, 1914, 62 and following. Pfeiffer, H., Das Problem der Eiweissanaphylaxie, Jena, 1910; Die Arbeitsmethoden bei Versuchen über Anaphylaxie, *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1911, v, 525. Zunz, E., Recherches sur le pouvoir protéoclastique du sang au cours de l'anaphylaxie (1re à 3e communications), *Z. Immunitätsforsch., Orig.*, 1913, xvii, 279.

[13] Zunz, E., and György, P., Recherches sur le pouvoir protéoclastique du sang au cours de l'anaphylaxie, (4e communication), *Z. Immunitätsforsch., Orig.*, 1914–1915, xxiii, 402.

[14] Bordet and Zunz, Le sérum traité par l'agar épuré de son azote (pararabine) se protéolyse-t'-il, *Z. Immunitätsforsch., Orig.*, 1914–15, xxiii, 49.

ent symptoms of anaphylactic shock produced in the two species of animals.

Arthus[15] was able, in the course of numerous experiments, to assure himself of the innocuousness of intravenous injections of considerable quantities of horse serum in normal rabbits. However, the introduction of horse serum into the circulatory system of rabbits, which had first received subcutaneous serum injections, led to a series of general symptoms; fall of arterial pressure, anuria, accelerated respiration, and expulsion of a considerable number of scybala. It is easy to determine from the technique used by Arthus whether or not one is dealing with symptoms of anaphylactic shock.

We also employed this method to determine whether or not horse serum kept 2 hours at 38°C. on agar and treated in the manner indicated by Bordet in his experiments with guinea pigs, produces anaphylactic shock in normal rabbits.

Technique.

The suspension of agar was prepared as follows: 1 gm. of agar was dissolved by heating in 200 cc. of physiological salt solution. This solution was distributed in test-tubes in portions of 10 cc., sterilized, and sealed. Upon cooling, a soft jelly is formed. Vigorous shaking will transform this jelly into a homogeneous suspension of agar which keeps well.

To 50 cc. of horse serum were added 10 cc. of a suspension of agar; *i.e.*, five parts of serum to one part of agar. In addition we prepared the four following control mixtures:

1. 50 cc. of fresh horse serum plus 10 cc. of physiological salt solution.
2. 50 cc. of physiological salt solution plus 10 cc. of a suspension of 0.5 per cent agar.
3. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of physiological salt solution.
4. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of a suspension of 0.5 per cent agar.

The five mixtures were kept in well corked Erlenmeyer flasks for 2 hours at 38°C. At the end of this period, each solution was submitted first to rapid centrifugalization and then to filtration. Thus the agar contained in the test mixture and in the second and fourth of the control mixtures was removed.

The liquids were injected into rabbits in the marginal vein of the ear. The

[15] Arthus, M., La séro-anaphylaxie du lapin, *Arch. de physiol.*, 1908–09, vii, 471; 1910, ix, 156.

respiration was registered by Verdin's recording tambours, connecting with a Marey tambour; and the carotid pressure by Verdin's registering hemadynamometer. Franck's cannula was employed. As an anticoagulant a solution of 65 gm. of sodium bicarbonate and 15 gm. of magnesium sulphate in 1,000 cc. of water was employed.

In order to estimate the coagulability we discarded the first few cubic centimeters of carotid blood, contaminated by mixture with the anticoagulant solution. The blood is collected from the carotid directly into a porcelain vessel, without touching the wound. The time necessary for the formation of the first fibrin floccules and for complete coagulation was recorded.

EXPERIMENTAL.

We performed four series of tests, each with a fresh sample of horse serum. In the first series we prepared but one control mixture, of normal serum and physiological salt solution.

First Series of Experiments.

Experiment 1.—A rabbit weighing 1,800 gm. received an intravenous injection of 10 cc. of control serum (normal horse serum plus physiological salt solution, kept 2 hours at 38°C.); 8 minutes later 10 cc. of the filtered test mixture (serum plus suspension of agar) were injected. This dose corresponds to 5.56 cc. per kilo of control serum and of serum treated with agar. Table I gives the results of arterial pressure and respiratory rate in the experiments.

TABLE I.

Injection of Normal Horse Serum and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.0	60	4	8.7	67
½	9.0	68	5	8.7	57
1	8.9	65	6	8.6	55
2	8.9	63	7	8.8	62
3	8.8	69			

Injection of Filtrate of Serum Treated with Agar.

½	9.0	63	13	7.6	61
1	9.0	64	14	7.7	60
2	8.6	61	15	7.7	63
3	8.2	58	16	7.4	58
4	8.3	62	17	7.3	61
5	8.2	61	18	7.2	63
6	8.4	62	19	7.3	60
7	8.2	67	20	7.2	58
8	8.2	64	21	7.3	60
9	8.1	54	22	7.3	58
10	8.0	55	23	7.4	53
11	8.0	61	24	7.5	61
12	7.6	62	25	7.5	58

The intravenous injection of control serum caused a gradual slow fall in the arterial blood pressure. In 6 minutes it fell 0.4 cm. of mercury, and then tended to rise again. After injecting serum treated with agar the fall in pressure was also gradual, but much greater. It amounted to 1.8 cm. of mercury in 18 minutes. The arterial pressure remained stationary for a time, and rose again slowly. The respiration showed no distinct changes after either the first or second injection. There was no defecation.

Blood taken from the carotid 26 minutes after the second injection showed the first fibrin formation at the end of 18 minutes. Coagulation was complete 4 minutes later; that is, 22 minutes after drawing the blood.

Experiment 2.—A rabbit weighing 1,750 gm. received intravenously an injection of 5 cc. of control serum (normal horse serum plus physiological salt solu-

tion kept 2 hours at 38°C.). 8 minutes later 5 cc. of the filtered test mixture (serum plus agar suspension) were given. This dose corresponds to 2.86 cc. per kilo of control serum and of serum treated with agar. (Table II.)

TABLE II.

Injection of Normal Horse Serum and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.6	62	4	9.6	68
½	9.9	68	5	9.7	65
1	9.9	60	6	9.6	69
2	9.8	67	7	9.6	65
3	9.6	67			
Injection of Filtrate of Serum Treated with Agar.					
½	9.9	68	11	9.0	56
1	9.6	65	12	9.0	58
2	9.3	68	13	9.2	62
3	9.1	62	14	9.3	65
4	9.0	63	15	9.3	65
5	9.0	57	16	9.1	67
6	8.9	67	17	9.1	63
7	8.8	62	18	9.2	63
8	8.8	63	19	9.3	66
9	9.0	57	20	9.4	60
10	9.0	62	21	9.4	65

The injection of control serum did not lead to a noticeable modification of the respiratory rate or of the arterial pressure, except for a slight temporary increase of the pressure, 0.3 cm. of mercury. The injection of serum treated with agar had also no effect on the respiration. The arterial pressure rose temporarily 0.3 cm. of mercury, then it fell in 7 minutes 0.8 cm. of mercury, as compared with the initial level. No defecation took place after either injection.

Blood taken from the carotid 22 minutes after the injection of serum treated with agar showed the first fibrin formation at the end of 8 minutes; coagulation was complete in 7 minutes more; that is, 15 minutes after the withdrawal of the blood.

In this first series of experiments we noted an effect of the serum treated with agar on the arterial pressure. This effect was very

slight with a dose of 2.86 cc. of serum per kilo, and more pronounced with 5.56 cc. per kilo.

Second Series of Experiments.

Experiment 3.—A rabbit weighing 1,620 gm. received an intravenous injection of 5 cc. of serum treated with agar; that is, 3.09 cc. per kilo. (Table III.)

TABLE III.

Injection of Filtrate of Serum Treated with Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.9	51	17	6.4	53
½	9.9	50	18	6.4	53
1	9.9	45	19	6.4	52
2	8.1	45	20	6.3	48
3	7.9	50	21	6.4	50
4	7.5	55	22	6.5	50
5	6.4	49	23	6.5	50
6	5.8	49	24	6.4	50
7	5.8	48	25	6.5	61
8	6.5	59	26	6.6	55
9	6.3	47	27	6.7	52
10	6.4	50	28	6.6	55
11	6.6	47	29	6.7	51
12	6.6	48	30	6.7	48
13	6.3	56	31	6.8	48
14	6.2	60	32	6.4	48
15	6.2	53	33	6.6	53
16	6.3	58			

After the injection of serum which had been kept for 2 hours at 38°C. in contact with agar, the arterial pressure fell 4.1 cm. of mercury in 6 minutes. The pressure rose again 0.7 cm. 8 minutes after the injection and remained, with some variation, near this level until the end of the experiment. The respiration did not undergo any actual modifications. There was an expulsion of thirty scybala shortly after the injection.

The carotid blood, drawn 34 minutes after the injection, showed the first fibrin formation at the end of 13 minutes; coagulation was complete 4 minutes later, or 17 minutes after the drawing of blood.

Experiment 4.—A rabbit weighing 1,810 gm. was injected intravenously successively with 5 cc. of the following liquids, 2.76 cc. of each per kilo. The order of injections was as follows:

1. Serum and physiological salt solution.
2. Filtrate of agar and physiological salt solution.
3. Filtrate of serum, which had been heated to 56°C., and of agar.
4. Serum previously heated to 56°C. and physiological salt solution.
5. Filtrate of serum and agar.

About half a minute was required for each injection. (Table IV.)

TABLE IV.

Injection of Serum and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.4	83	4	10.6	89
½	12.2	98	5	10.3	100
1	11.6	89	6	10.2	112
2	11.4	92	7	10.3	99
3	11.1	90	8	10.6	104
Injection of Filtrate from Control Mixture of Agar and Physiological Salt Solution.					
½	10.0	102	5	9.6	92
1	10.0	98	6	9.4	94
2	10.1	95	7	9.3	83
3	9.8	98	8	9.4	84
4	9.7	95	9	9.6	78
Injection of Filtrate of Serum Heated to 56° C. and Agar (Control).					
½	9.7	77	5	9.0	56
1	9.6	76	6	9.0	67
2	9.0	62	7	9.0	67
3	9.0	52	8	9.3	68
4	8.9	54	9	9.5	70
Injection of Serum Heated to 56°C. and Physiological Salt Solution (Control).					
½	9.6	72	5	9.7	73
1	9.7	70	6	9.7	75
2	9.8	69	7	9.6	74
3	9.8	75	8	9.6	72
4	9.7	76			

TABLE IV—*Concluded.*

Injection of Filtrate of Serum Treated with Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
$\frac{1}{2}$	9.8	76	14	7.0	95
1	9.6	83	15	7.0	97
2	8.8	81	16	6.7	109
3	7.1	68	17	6.4	107
4	7.6	67	18	5.0	102
5	7.6	63	19	5.6	111
6	7.4	67	20	5.4	105
7	7.2	72	21	5.9	105
8	7.4	73	22	5.9	97
9	7.4	82	23	6.2	100
10	7.3	72	24	5.4	102
11	7.1	74	25	5.4	93
12	7.2	87	26	6.0	90
13	7.0	97	27	6.0	82

The arterial pressure rose considerably (1.8 cm. of mercury) immediately after the intravenous injection of the control serum kept 2 hours at 38°C. Subsequently it returned to its normal level and even fell somewhat (0.2 cm. of mercury) below normal toward the 6th minute, again rising slightly (0.2 cm. of mercury) above its original level 8 minutes after the injection.

The introduction into the circulation of physiological salt solution treated with agar caused an immediate fall of the arterial pressure, amounting to 1.3 cm. of mercury in 7 minutes. Then the pressure commenced to rise again.

The serum previously heated to 56°C., then treated with agar, caused a fall in the arterial pressure of 0.7 cm. of mercury in 4 minutes. The pressure rose slightly after the 7th minute following the injection.

This serum heated to 56°C., and kept for 2 hours at 38°C., had no actual effect on the arterial pressure, for we cannot ascribe to it the slight increase of 0.2 to 0.3 cm. of mercury, which would possibly also have taken place without the last injection, and which stopped before the arterial pressure had regained the level at which it was before the third injection.

The action on the arterial pressure of serum treated with agar was much more marked in the rabbit of Experiment 4 than that of the different control liquids. It consisted of a gradual fall. The pressure attained its minimum 18 minutes after the injection. At this moment it had fallen 4.8 cm. of mercury. Then it rose again about 1 cm. and remained at this level, with some fluctuations, until the end of the experiment.

If we compare the effects of the various intravenous injections in the rabbit of Experiment 4, and the results obtained in the rabbits of Experiments 3 and 4 after the intravenous injection of serum treated with agar, it is evident that this serum has acquired, after the treatment, the power of producing in a normal animal a fall of arterial pressure as marked as the one induced in a sensitized animal by intravenous injection of sensitizing serum. Prior to this treatment horse serum had different properties, since it caused a rise in the arterial pressure. After being exposed 30 minutes to 56°C. and treated with agar, the serum loses its power of causing any considerable fall in the arterial pressure. The inactivated serum has no effect on the pressure. It is of interest to note the relatively slight fall in pressure, caused by the physiological salt solution kept 2 hours at 38°C. on agar. This phenomenon is sometimes more pronounced, as seen in the third and fourth series of experiments.

We shall now consider the respiratory movements. The results obtained in the course of Experiment 4 may be summed up as follows: acceleration of respiratory rate was as great after the injection of serum treated with agar (maximum at the end of 19 minutes) as after the injection of control serum kept 2 hours at 38°C. (maximum at the end of 6 minutes); gradual slowing after injection with physiological salt solution previously treated with agar; slowing during 3 minutes following the injection of serum previously heated to 56°C., then treated with agar, and finally a return to the initial rate; no actual modifications after the injection of serum heated to 56°C. and then kept for 2 hours at 38°C. with the addition of physiological salt solution instead of agar suspension.

There was no expulsion of feces after the first four injections. The introduction into the blood of serum treated with agar caused abundant micturition, followed by rapid expulsion of fifty-five scybala.

The blood drawn 28 minutes after the injection of serum treated with agar showed the first fibrin formation at the end of 18 minutes. Coagulation was completed in 3 minutes more, 21 minutes after drawing the blood. The rabbits died within 2 to 14 hours after the last injection.

Experiments 3 and 4 lead to the conclusion that by keeping normal horse serum for 2 hours at 38°C. in the presence of agar, which has been extracted by centrifugalization and filtration, this serum acquires the power to induce in a normal rabbit certain symptoms of anaphylactic shock; namely, a marked fall in the arterial pressure which persists for a long time, and an abundant expulsion of feces. The serum, previously exposed for 30 minutes to 56°C., does not acquire this property after being treated with agar.

Third Series of Experiments.

Experiment 5.—A rabbit weighing 2,500 gm. received intravenously an injection of 10 cc. of serum treated with agar, which corresponds to 4 cc. per kilo. (Table V.)

TABLE V.

Injection of Filtrate of Serum Treated with Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	8.2	78	11	8.3	78
½	8.8	61	12	8.5	73
1	8.6	61	13	8.6	75
2	8.7	63	14	8.7	64
3	9.0	66	15	8.8	68
4	9.6	56	16	8.9	62
5	9.4	72	17	9.0	70
6	8.7	80	18	9.0	71
7	8.4	80	19	9.0	77
8	8.2	79	20	9.0	69
9	8.4	84	21	9.0	69
10	8.3	78			

With a dose of 4 cc. per kilo the intravenous injection of serum treated with agar induced, in the first place, an increase in the arterial pressure, which reached its maximum (1.4 cm. of mercury) 4 minutes

after the injection. The arterial pressure then returned to its initial maximum level at the 8th minute following the injection, then increased 0.8 cm. of mercury up to the 17th minute after the injection, remaining at this level until the end of the experiment.

The respiration diminished in frequency during the first 4 minutes following the introduction into the blood of serum treated with agar; then it rose to the initial figure where it remained, showing slight variations in both directions (slight accelerations and retardations). There was an expulsion of sixteen scybala immediately after the injection.

The blood drawn from the carotid 12 minutes after the injection showed the first fibrin formation at the end of 7 minutes. Coagulation was completed 15 minutes after the withdrawal of blood.

Experiment 6.—A rabbit weighing 2,300 gm. was injected intravenously with 5 cc. of serum treated with agar, which corresponds to 2.17 cc. per kilo. (Table VI.)

TABLE VI.

Injection of Filtrate of Serum Treated with Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	11.8	123	7	9.7	145
$\frac{1}{2}$	12.2	132	8	9.9	121
1	11.8	107	9	9.9	113
2	11.6	108	10	10.0	130
3	11.4	91	11	10.1	105
4	10.5	109	12	10.1	107
5	10.2	117	13	10.1	100
6	9.6	114	14	10.1	95

With a dose of 2.17 cc. per kilo, the intravenous injection of serum treated with agar caused, after a temporary increase in the arterial pressure immediately after the injection, a gradual fall up to the 6th minute after this injection. At this moment the arterial pressure had fallen 2.2 cm. of mercury below the initial level. Then the pressure rose again 0.5 cm. of mercury in 5 minutes, where it remained up to the 20th minute after the injection.

The respiration, which was very rapid before the injection, first

underwent a slight acceleration, followed by a retardation until the 3rd minute after the injection, then an acceleration through the 7th minute, and slight retardations and accelerations. After the injection there was an expulsion of twenty-five scybala.

The blood extracted from the carotid 15 minutes after the injection first showed fibrin formation at the end of 15 minutes. Coagulation was complete 33 minutes after the injection.

Experiment 7.—A rabbit weighing 2,800 gm. received intravenously an injection of 3 cc. of serum treated with agar, or 1.07 cc. per kilo; then 12 minutes later 5 cc. of the same serum treated with agar, or 1.78 cc. per kilo. (Table VII.)

TABLE VII.

Injection of Filtrate of Serum Treated with Agar, 1.07 cc. per Kilo.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.0	39	6	8.2	72
$\frac{1}{2}$	9.5	47	7	8.1	77
1	9.6	36	8	8.0	86
2	9.5	44	9	7.9	84
3	8.9	67	10	7.9	76
4	8.7	60	11	7.9	78
5	8.3	60			

Injection of Filtrate of Serum Treated with Agar, 1.78 cc. per Kilo.

$\frac{1}{2}$	8.1	67	5	7.7	60
1	7.7	49	6	7.7	77
2	7.7	55	7	7.6	68
3	7.7	63	8	7.5	60
4	7.9	65			

The first injection of serum treated with agar, with a dose of 1.07 cc. per kilo, caused within 9 minutes a fall in the arterial pressure corresponding to 2.1 cm. of mercury. During the same time the respiration increased in frequency and reached its maximum, which was more than double the initial number, at the end of 8 minutes.

The second injection of serum treated with agar had hardly any influence on the arterial pressure. Immediately after the injection a slight increase of 0.2 cm. of mercury was produced, then the pres-

sure fell slowly. The respiration, remaining always above the initial frequency, slowed down somewhat, especially 1 or 2 minutes after this new injection. There was no expulsion of feces after the first, and an expulsion of only four scybala after the second injection.

Coagulation, however, was markedly retarded. The blood taken from the carotid 9 minutes after the second injection did not commence to show the first fibrin formation until the end of 20 minutes. Coagulation was not complete until 45 minutes after the extraction of blood.

Experiment 8.—A rabbit weighing 1,450 gm. received an intravenous injection of 5 cc. of the control mixture of serum and physiological salt solution, and 7 minutes later the same quantity of filtrate obtained from the control mixture of physiological salt solution and agar, and finally, 13 minutes after this second injection, 5 cc. of filtrate from serum treated with agar. Each of these injections corresponds to 3.45 cc. per kilo. (Table VIII.)

TABLE VIII.

Injection of Serum and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	8.1	36	3	8.4	39
½	8.9	35	4	8.4	38
1	8.3	38	5	8.3	38
2	8.4	34	6	8.2	41

Injection of Filtrate from Control Mixture of Agar and Physiological Salt Solution.

½	8.2	39	7	5.5	43
1	8.0	37	8	5.3	40
2	8.5	43	9	5.7	43
3	7.0	39	10	5.8	42
4	6.7	40	11	6.0	42
5	6.3	41	12	6.1	42
6	5.6	40			

Injection of Filtrate of Serum Treated with Agar.

½	6.9	42	3	6.6	36
1	5.8	53	4	6.4	39
2	6.3	59	5	6.7	38

The arterial pressure rose 0.8 cm. of mercury immediately after the intravenous injection of serum kept for 2 hours at 38°C. in the presence of physiological salt solution. Then it fell 0.6 cm. of mercury and remained a little higher than the initial pressure. It fell until the 8th minute after the introduction into the blood of filtrate from the mixture of physiological salt solution and agar, then it began to rise; the fall amounted to 2.9 cm. of mercury. We encountered here the same phenomenon, though more pronounced, as in Experiment 4. The injection of serum treated with agar, with a dose of 3.45 cc. per kilo, first caused the arterial pressure to rise 0.8 cm. of mercury. This increase was immediately followed by a fall of 1.1 cm. of mercury, then the pressure rose almost to the same level as half a minute after the last injection. The impression obtained is that two opposite actions are produced, and prevent the sharp fall in the arterial pressure which would have been caused by the intravenous injection of serum treated with agar, when the injected dose is 1.07 to 2.17 cc. per kilo (Experiments 7 and 6).

The frequency in respiration was not modified after the intravenous injection either of the control serum or of the filtrate from the mixture of agar and physiological salt solution. It showed a slight and momentary increase after the introduction into the blood of the filtrate of serum treated with agar; then the respiratory rate returned to normal. There was no defecation after the first two injections, but after the last injection there was an expulsion of ten scybala.

Experiment 9.—A rabbit weighing 2,250 gm. received into the marginal vein of the ear 5 cc. of filtrate from the control mixture of physiological salt solution and agar. This dose corresponds to 2.22 cc. per kilo. (Table IX.)

TABLE IX.

Injection of Filtrate from Control Mixture of Physiological Salt Solution and Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.6	57	5	4.6	89
½	11.2	60	6	5.0	100
1	10.5	53	7	6.2	98
2	10.1	48	8	7.2	95
3	10.1	56	9	8.0	95
4	3.5	75	10	8.0	80

With this rabbit and the dose employed, the filtrate from the control mixture of physiological salt solution and agar caused, after a slight momentary increase, a rapid fall in the arterial pressure 4 minutes after the injection. The fall amounted to 7.1 cm. of mercury, much more than that obtained with serum treated with agar in this series of experiments. The pressure then rose rapidly, contrary to that observed after the intravenous injection of serum kept for 2 hours at 38°C. in the presence of agar.

The respiration showed, after the 3rd minute following the injection, an acceleration, which reached its maximum 6 minutes after the injection; then the respiration gradually slowed to a slight extent, yet without reaching the initial rate, within 10 minutes after the injection.

There was no expulsion of feces. The blood extracted from the carotid 11 minutes after the injection showed the first fibrin formation at the end of 10 minutes. Coagulation was completed 5 minutes later, 15 minutes after withdrawing the blood.

In the third series of experiments the results were less clear than in the second, because the intravenous injection of filtrate, extracted from the control mixture of agar and physiological salt solution, caused a fall in the arterial pressure more intense, yet more temporary than that caused by appropriate doses of filtrate extracted from serum treated with agar. Besides, this control mixture sometimes caused respiratory acceleration.

Fourth Series of Experiments.

Experiment 10.—A rabbit weighing 2,100 gm. was injected intravenously with 3 cc. of serum treated with agar, 1.43 cc. per kilo; 8 minutes later an injection was given of 6 cc. of the same serum, or 2.86 cc. per kilo. (Table X.)

TABLE X.

Injection of Filtrate of Serum Treated with Agar, 1.43 cc. per Kilo.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.4	150	4	10.0	150
½	10.4	150	5	10.0	140
1	10.4	140	6	10.0	160
2	10.3	140	7	10.1	145
3	10.2	150			

Injection of Filtrate of Serum Treated with Agar, 2.86 cc. per Kilo.

½	10.0	140	10	9.4	121
1	9.4	140	11	9.4	123
2	9.5	130	12	9.4	110
3	10.0	140	13	9.4	110
4	9.9	148	14	9.4	102
5	9.8	139	15	9.5	125
6	9.5	130	16	9.3	123
7	9.5	107	17	9.3	105
8	9.4	129	18	9.3	110
9	9.5	133			

The arterial pressure fell 0.4 cm. of mercury 4 minutes after the first injection, and 0.7 cm. of mercury within 16 minutes after the second injection, or 1.1 cm. in all. The respiration did not undergo any modifications after the first injection, but from the 6th minute after the second injection it tended to fall and reached its minimum at the 14th minute after the second injection. After the second injection there was an expulsion of sixteen scybala.

The blood, drawn from the carotid 19 minutes after the second injection, showed the first fibrin formation at the end of 25 minutes. Coagulation was complete 10 minutes later, or 35 minutes after drawing the blood.

Experiment 11.—A rabbit weighing 2,200 gm. received an intravenous injection of 5 cc. of serum treated with agar, which corresponds to 2.27 cc. per kilo. (Table XI.)

TABLE XI.

Injection of Filtrate of Serum Treated with Agar.

Time after injection.	Arterial pressure.	Respiratory rate	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	13.3	67	10	10.7	46
$\frac{1}{2}$	12.4	68	11	10.5	49
1	12.2	68	12	10.3	44
2	12.1	86	13	10.0	46
3	12.0	102	14	10.0	38
4	11.7	90	15	10.0	39
5	11.9	96	16	10.0	39
6	11.6	74	17	10.0	38
7	12.0	69	18	10.0	39
8	11.7	67	19	10.0	42
9	11.4	52			

The arterial pressure fell 3.3 cm. of mercury in 13 minutes, remaining at this level for at least 6 minutes more. The respiration increased in frequency from the 1st minute, reaching its maximum 3 minutes after the injection. Then the number of respiratory movements gradually decreased, reaching the initial rate 6 or 7 minutes after the injection, and continued falling until the 14th minute after the introduction into the blood of serum treated with agar, showing no further modifications till the end of the experiment. After the injection violent movements of the intestines were observed through the abdominal wall, and there was an expulsion of sixty-nine scybala.

The blood, extracted from the carotid 20 minutes after the injection, showed the first fibrin formation at the end of 20 minutes. Coagulation required 5 more minutes and was complete 25 minutes after drawing.

Experiment 12.—A rabbit weighing 2,550 gm. received an intravenous injection of 5 cc. of filtrate from the control mixture of physiological salt solution and agar, then 14 minutes later 5 cc. of control serum were injected, and finally, 19 minutes after, 5 cc. of serum which had been kept for 30 minutes at 56°C. and then treated with agar. With each injection 1.96 cc. of liquid per kilo were introduced into the blood. (Table XII.)

TABLE XII.

Injection of Filtrate from Control Mixture of Physiological Salt Solution and Agar.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	12.7	110	7	12.6	52
½	12.5	95	8	12.0	54
1	12.2	100	9	11.8	48
2	12.1	105	10	11.6	46
3	11.8	94	11	11.8	40
4	11.8	94	12	12.0	42
5	11.6	87	13	12.6	42
6	11.4	80			

Injection of Serum and Physiological Salt Solution (Control).

½	12.2	46	10	11.6	46
1	12.2	50	11	11.5	44
2	12.2	51	12	11.6	42
3	12.0	40	13	11.6	45
4	12.0	45	14	11.6	45
5	12.4	46	15	11.7	45
6	11.8	48	16	11.8	46
7	11.8	44	17	12.0	47
8	11.5	41	18	12.2	46
9	11.7	47			

Injection of Filtrate of Serum Heated to 56° C. and Treated with Agar.

½	12.1	44	5	12.2	42
1	12.1	42	6	12.3	45
2	12.1	46	7	12.3	45
3	12.1	43	8	12.3	45
4	12.1	45			

The arterial pressure fell 1.3 cm. of mercury 6 minutes after the intravenous injection of filtrate from the mixture of agar and physiological salt solution, and then returned to normal. The injection of control serum produced a fall in the arterial pressure corresponding to 1.1 cm. of mercury in 8 minutes; the pressure then rose slowly, and as late as 18 minutes after the injection it had not reached the initial level. The introduction at this time of serum heated to 56°C.

before being subjected for 2 hours to 38°C. in the presence of agar had no effect on the arterial pressure, which continued to rise slowly.

The respiration decreased in frequency under the influence of the filtrate obtained from the mixture of agar and physiological salt solution until the 11th minute after the injection. The respiratory rate showed no further material modifications, even after the injections—whether of the mixture of serum and physiological salt solution or of the filtrate from the mixture of serum and agar previously subjected to a temperature of 56°C. There was no defecation during the experiment.

Experiments 11 and 12 show a marked effect on the blood pressure by an appropriate dose of serum treated with agar. The fall was more pronounced and more persistent than under the influence of an analogous dose of filtrate from a control mixture of physiological salt solution and agar. In this series of experiments the introduction into the blood of control serum kept for 2 hours at 38°C. in the presence of physiological salt solution produced a slight fall in the arterial pressure, although in the second and third series this injection caused a temporary increase in the pressure.

DISCUSSION.

The experiments show that the intravenous injection of adequate doses of normal horse serum kept for 2 hours at 38°C. in the presence of agar and then freed from this agar through centrifugalization and filtration produces in normal rabbits a marked and prolonged fall in the arterial pressure. To produce this phenomenon to any marked degree, it is necessary to avoid an excess of the serum; for in such a case we should, on the contrary, experience a slight increase in the arterial pressure (Experiment 5). Nor should too small a dose of serum be employed; for in this case we should have only a slight fall in the arterial pressure (Experiments 2 and 10).

A second intravenous injection of serum treated with agar produces but a slight fall in the pressure (Experiments 7 and 10). The adequate dose of serum treated with agar varies in different sera and, to a less extent, in different rabbits.

In order to show clearly the effects of serum treated with agar upon the arterial pressure of normal rabbits, it is necessary to inject into

the rabbits increasing doses of serum: 1.00, 1.25, 1.50, 1.75, and 2.00 cc. per kilo, and, according to the results obtained, smaller or larger quantities. Each dose must be injected into two or three rabbits. When the proper dose per kilo is thus established, we proceed with tests using an equal dose of each control liquid in two or three normal rabbits. Likewise we should employ in the same animal only one kind of liquid (serum treated with agar or one of the control mixtures). Unfortunately, we did not have at our disposal the number of animals necessary for the technique outlined above.

We wish to emphasize the fact that it is preferable to employ, as in the experiments with guinea pigs, homologous serum; that is, rabbit serum.

Our results are supported by those of Bordet and Zunz and of other investigators who have studied the action of serum kept at 38°C. in the presence of agar and then separated from the latter material.

In normal rabbits this serum produces the same modifications in the arterial pressure as those produced by intravenous injections of horse serum in sero-anaphylactized rabbits. Before treatment with agar sometimes this is not the case and even a slight fall in the pressure may be produced. The filtrate from the control mixture of agar and physiological salt solution at times produces a pronounced fall of the arterial pressure, but this fall differs from that caused by serum treated with agar, since the pressure returns relatively rapidly to its initial level in the first case, whereas in the second the pressure remains for a long time at quite a low level.

The modifications in respiration are not very pronounced after the intravenous injection of serum treated with agar. The respiratory rate, nevertheless, increases to a certain degree, four times out of twelve, after this injection (Experiments 4, 7, 8, and 11).

This occurred once out of four times after the injection of filtrate from the mixture of agar and physiological salt solution (Experiment 9), and once out of five times after the injection of control serum kept for 2 hours at 38°C. in the presence of physiological salt solution (Experiment 4).

Serum treated with agar has a marked effect on the intestinal movements. Expulsion of feces never occurred under the influence of the control mixtures.

Coagulation in the carotid blood was at times very much retarded (Experiment 7) under the influence of intravenous injection of serum treated with agar. At other times the phenomenon was less pronounced. With the same amount of the same kind of serum, the intensity corresponded to the degree of the fall in the arterial pressure. There was little or no retardation in coagulation, and the pressure showed little or no fall. A marked relation seemed to exist between these two phenomena.

The intravenous injection of filtrate from the mixture of agar and physiological salt solution did not cause any obvious modifications in the coagulation of the carotid blood, even though the arterial pressure underwent a pronounced fall but one of short duration (Experiment 9). In this respect, the effects of this injection were distinctly different from those due to the introduction of serum treated with agar into the circulatory system of normal rabbits.

Horse serum exposed to a temperature of 56°C. for 30 minutes and treated with agar does not cause, in normal rabbits, a pronounced and lasting fall in the arterial pressure and the other phenomena produced under the influence of the intravenous injection of the normal serum kept for 2 hours at 38°C. in the presence of agar.

SUMMARY.

The intravenous injection of horse serum, kept for 2 hours at 38°C. in the presence of one-fifth of its volume of a suspension of 0.5 per cent agar in physiological salt solution and then separated from the agar by centrifugalization and filtration, produces in normal rabbits in adequate doses a considerable and prolonged fall in the blood pressure, expulsion of feces, a diminished coagulability in the carotid blood, and at times accelerated respiration; that is, the various symptoms observed after the intravenous injection of horse serum in a sero-anaphylactized rabbit.

Horse serum previously kept for 30 minutes at 56°C. and then treated with agar in the manner described above, when injected intravenously into a normal rabbit will have no more effect on the arterial pressure, on the intestinal movement, on the respiratory rate, or the coagulation of arterial blood than the introduction of horse serum into the veins of a normal rabbit.

AN EXPERIMENTAL STUDY OF CIRCUMSCRIBED DILATION OF AN ARTERY IMMEDIATELY DISTAL TO A PARTIALLY OCCLUDING BAND, AND ITS BEARING ON THE DILATION OF THE SUBCLAVIAN ARTERY OBSERVED IN CERTAIN CASES OF CERVICAL RIB.*

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PLATES 16 TO 19.

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No one, since Deitmar,[1] has attempted to collate the cases of dilation of the subclavian artery associated with cervical rib. Deitmar cites five cases (Adams, Coote, Poland, Baum, and von Heinecke), including one (von Heinecke's) which I have tabulated as doubtful. Streissler's review[2] is perhaps the fullest in the literature on the subject of cervical rib. Although it appeared less than 3 years ago no addition is made by this author to Deitmar's list.

From a careful study, in the original, of the reports of 716 cases of cervical rib I find that aneurysm or dilation of the subclavian artery was noted in 27 or more of them, including six (Mayo, Murphy, Russel, von Heinecke, Galloway, and Seymour) in which the surgeon believed that the vessel was abnormally large, and two (Karg and Halsted) in which the aneurysm appeared promptly after removal of the supernumerary rib. There may be numerous other instances of dilation of the subclavian associated with cervical rib—cases in which the amount of arterial expansion could not be determined in the lack of a standard of comparison.

The Dilation of the Subclavian Artery Is Distal to the Line of Constriction Made by the Rib and the Scalenus Anticus Muscle.

As to the cause of these aneurysms there is much conjecture. The comment has frequently been made that their occurrence would be

* It is possible in the limits of the permissible space to present the results of the work of Dr. Reid and myself in merest outline. A full account will probably appear next year in the *Reports of the Johns Hopkins Hospital*.

[1] Deitmar, J., Inaug. Diss., Erlangen, 1907.

[2] Streissler, E., *Ergebn. Chir. u. Orthop.*, 1913, v, 280.

comprehensible if they appeared on the proximal instead of the peripheral side of the compression. No one has remarked that dilation central to the site of pressure might be even more difficult to comprehend.

The suggestions which have been offered in the effort to explain the phenomenon are as follows: (1) weakening of the wall of the artery from erosion or other trauma; (2) variable or intermittent pulse pressure; (3) vasomotor and vasa vasorum disturbances.

In 1906 Dr. E. H. Richardson and I made the observation that after partial occlusion of the thoracic aorta the maximum pressure may be permanently lowered and the minimum pressure actually increased distal to the constricting band of metal. This discovery was verified by Dr. Dawson on one of my dogs (No. 96).

Dog. 96. Partial Occlusion of the Thoracic Aorta.

Operation, May 22, 1906. Killed 7 months later.

	Maximum pressure.	Mean pressure.	Minimum pressure.	Pulse pressure.
Femoral	116	93	88	28
Carotid	160	113	83	77

The dilation of the artery observed in arteriovenous fistula might, I thought, have a bearing on the interpretation of the aneurysms in cases of cervical rib. "May not both phenomena," I asked myself, "be due to degenerative changes in the arterial wall consequent upon lowered pressure?"

Inasmuch as dilation of the subclavian artery has, relatively to the number of cases of cervical rib, so seldom been observed, it seemed that if it were due merely to the lowered pulse pressure, then only a very definite absolute or relative amount of reduction of the systolic pressure would suffice to produce it. It was realized, also, that even if the amount of reduction necessary to accomplish the desired result could be determined it could not be constantly maintained, inasmuch as the peripheral resistance becomes, in great measure, rapidly restored.

For a number of years, in the course of various experiments in partial occlusion of the arteries, I had somewhat in view the chance of there being produced beyond the point of constriction a dilation of

the artery analogous to that which had been observed in cases of cervical rib.

The Degree of Constriction and the Period of Time Required for the Production of the Dilation.

2 years ago when, after many trials, I had altogether despaired of having the hope realized, I was startled, on examining the abdomen of a dog whose aorta had been constricted for 5 months and 20 days, to see that each of the branches of trifurcation was dilated almost to the size of the main aortic trunk. About to leave town for the summer, I communicated the finding to Dr. Reid, asking him promptly to repeat the experiment, as precisely as possible. In the autumn we expectantly laparotomized three dogs upon which Dr. Reid had operated in the early summer and were disappointed to find that no change had taken place in the size of the aorta or its three terminal branches. Confident that there could have been no error in the original observation, I constricted the abdominal aorta, to various degrees, just above the trifurcation in twenty dogs and, at intervals, in the course of the winter, explored and reexplored the abdominal cavities, but with negative result, at least as concerned dilation.

Finally, on opening the abdomen of our last dog (No. 7) we found the dilation which we sought. The result is depicted in Figs. 1 to 4. The occlusion of the aorta in this case made 6 months and 19 days previously was almost complete, just as it had been in the one successful case of the foregoing year—I might say, of all the foregone years.

The pressure exercised by the band in this instance had been sufficient to lessen greatly, if not to obliterate, for a few moments at least, the palpable thrill produced by the constriction, but not enough completely to shut off the palpable pulse. With this observation as a fresh incentive, Dr. Reid and I have continued the experiments during the current academic year with encouraging results.

If the occlusion must be so nearly complete in order to effect a well pronounced dilation, it will explain not only the difficulty we have had in producing it in dogs, but also the fact that it has been observed so seldom in the human subject from compression of the subclavian

artery by a cervical rib. For when in dogs the aortic pulse is occluded beyond the stage of palpable thrill the lumen is in danger of becoming obliterated, as by the formation of a cylindrical fibrous cord beneath the band, and thus cancelling the experiment; and in the cervical rib cases we may assume argumentatively that the subclavian artery compressed to the stage sufficient to produce an aneurysm might become thickened by sclerotic changes in the time required for the pronounced manifestation of a dilation. Thus, a moderate dilation, present for a brief period prior to occlusion of the subclavian, might be overlooked.

In dogs, a number of months must apparently elapse after the application of the band before a dilation in striking degree can occur. In the two cases (No. 7. 1913, and No. 7, 1914) to which reference has been made, 5 months and 20 days, and 6 months and 19 days, respectively, had elapsed. In the second of these, a dilation of the middle branch of the trifurcation of 2 mm., found at the expiration of 60 days, had increased to 4 mm. in the course of the following $4\frac{1}{2}$ months (Figs. 1 to 4). Other instances of like dilation are accurately represented in Figs. 5 to 11. The amount of constriction made in two of the illustrated cases (Dogs 3 and 15, Series II) is indicated by the concentric circles of Figs. 8 and 11.

We have found and abstracted reports of 716 instances of cervical rib, in great part from the original.

Clinical cases	525
Autopsies	91
Museum specimens	100
Total	716

		per cent
Cases with nerve symptoms alone	235	65.3
Cases with nerve and vascular symptoms	106	29.4
Cases with vascular symptoms alone	19	5.3
Total	360	

525 were clinical cases; 91, autopsy findings; and 100, museum specimens. 360 presented symptoms of pressure. Of these, 235 had nerve symptoms alone; 106, nerve and vascular; and 19, only vascular symptoms. Accordingly we have reports of 125 cases of cervical rib

in which vascular symptoms were noted. In 27 of these (21.6 per cent) an enlargement, fusiform, aneurysmal, or cylindrical, was observed, and, of these, in the majority, the disturbance of circulation was severe, 6 cases having gangrene of fingers on the affected side (Table I).

Of the thirty dogs with aortic constriction upon which this report is based there was pronounced dilation, for a short distance, of the vessels below the band in seven, or 23.3 per cent.

It is interesting to note the correspondence in the human (21.6) and canine (23.3) percentages. We must not, however, overvalue the result of this haphazard sort of comparison, for, as regards the human cases we have depended upon the impressions of surgeons, who, having no standards of comparison, having made no measurements, and not always being particularly concerned about the arterial feature of the case, may have overlooked or overestimated variations from the norm; and, as regards the thirty dogs, we observed, in addition to the seven designated as major dilations, ten minor ones. The seventeen dilations of all grades represent 56.6 per cent of the thirty dogs. The percentage is even greater if we include in our calculation only the dogs of the past year; and, for another year of experimentation, would probably be greater still.

We may, I think, conclude that the dilation of an artery produced experimentally is not due to any of the three factors proposed as causal for the aneurysm in cases of cervical rib.

1. *Vasomotor Paralysis.*—(*a*) The vasomotor nerves and the vasa vasorum are destroyed by the moderately constricting and totally occluding bands quite as surely as by those which, occluding almost totally, alone have produced the dilation. (*b*) Only a portion of the circumference of the subclavian artery is exposed to the pressure of the cervical rib and the scalenus anticus muscle, and hence only a fraction of the vasomotor nerves or vasa vasorum could be pressed upon.

2. *Trauma.*—(*a*) Usually, the dilation is fusiform and (*b*) distal to the rib. (*c*) Trauma is excluded as a factor in the experimental dilations.

3. *Inconstant Blood Pressure.*—(*a*) Patients suffering from the pressure-pain of cervical rib rarely make wide excursion-movements of the arm. (*b*) The degree of occlusion is constant in the experimentally constricted vessel.

When an arterial trunk is ligated it becomes occluded to the first proximal and first distal branches by a process of cell proliferation which ultimately reduces the artery to a fibrous strand.

Is There a Fall in Blood Pressure in the Dead Arterial Pocket Which May Anticipate and Possibly Be a Causative Factor in the Obliteration of This Portion of an Artery?

From observations which I have made on man and dogs I am quite sure that there may be a remarkable fall in blood pressure in what I have termed the "dead arterial pocket," while there is still little if any sign of diminution in the caliber of this portion of the vessel. For example, the right common carotid artery was ligated by the writer in a case of aneurysm of the external carotid. About 3 months later, in the course of an operation for the excision of the uncured aneurysm, the internal carotid, dead-pocketed between the circle of Willis and the carotid ventricle, was freely exposed for a considerable distance. It had lost its cylindrical form, being flat and tape-like, and, although evidently possessing a considerable lumen, seemed to be empty. When pricked, a few drops of blood oozed without pulse from the little cut. The artery was then resected between two ligatures. Its wall was thickened on one side (Fig. 12) but the lumenwas still perhaps three times that of a radial artery. Similar observations I have made twice on the external iliac artery of the dog after occlusion of this vessel at its origin from the aorta. In the approximately dead pocket between the aorta and the origin of the circumflex iliac and common trunk of the epigastric and obturator arteries the blood pressure must have been almost nil, because from a little slit made in the apparently normal arterial wall of the relatively empty external iliac artery the blood escaped very slowly in a tiny, almost pulseless jet about 1 cm. high; whereas, from the femoral artery, below the profunda, the blood spurted normally from a similar knife-prick.

Hence in an artery doomed to obliteration, it would seem that the blood pressure may be lowered before the occlusion process sets in—the lowered pressure being, perhaps, the immediate factor leading to the obliteration.

Consideration of the Cause of the Dilation.

Can these observations have any bearing upon the explanation of the dilation of the aorta above its trifurcation, of its triad branches,

and of the carotid, which we have occasionally observed in the dog distal to the partially occluding band?

The mechanical engineer knows the effect upon pressures of constricting a rigid tube through which water is being forced at a given pressure. At the site of the constriction, of the Venturi meter, the pressure is diminished and the velocity increased, whereas immediately beyond the constriction both the normal pressure and velocity are restored. This is not, however, true of the constricted arterial tube. Beyond the band the systolic pressure may be lowered, the diastolic pressure increased, and the pulse pressure greatly reduced for many months at least.

If the constriction of the aorta is very slight the effect upon the blood pressures is usually transient, the normal pressures being reestablished within a few hours or, indeed, minutes. But if the artery is constricted to the point of almost total occlusion the pulse pressure below the band, for a time almost nil, may remain lowered and the diastolic pressure, relatively or even actually, be increased. The blood stream in this case, passing with greater velocity and less pressure through the band prevents the obliteration of the artery to the nearest branch, the pocket being not a dead one as it is in the case of total obliteration. The blood in this pocket beyond the constriction streams in whirlpools, somewhat as in the vein and, also, as in the artery in arteriovenous fistula; the thrill, not palpable at first, later may be perceived with the finger; and the bruit, always audible with the stethoscope, becomes louder as the peripheral arterial resistance increases.

To these factors, then,—to the abnormal play of the blood in the relatively, as distinguished from the absolutely dead pocket and to the absence of normal pulse pressure, essential probably to the maintenance of the integrity of the arterial wall, we may have to look for the solution of our problem.

It is not, however, denied that the paralysis of the vasomotor nerves and the occlusion of the vasa vasorum may possibly play some part in the manifestation. The dilations produced experimentally, like those observed in cases of cervical rib, are, as I have said, circumscribed. We had conjectured that the delimitation of the dilation might be influenced or determined by the location of the branches of the affected artery beyond the constriction. In one instance,

however, we observed, just distal to a partially occluding band applied to the carotid low in the neck of a dog, a circumscribed dilation of this vessel, the branches of which had been tied and divided. In this case there was a long stretch of debranched and patulous carotid between its dilated portion and the base of the skull.

Do Intimal Surfaces Brought Gently in Contact Unite, and What Is the Process by Which Obliteration of an Artery Takes Place after Occlusion by Band or Ligature?

The intimal surfaces of arteries brought intact in apposition whether by ligature or by band never, in my experience, have united. This statement will be received sceptically, for it is at variance with the quite universally accepted view, that uncrushed intimal surfaces if brought gently in contact adhere and thus occlude the artery. In the ligation of the larger arteries we have been taught to use heavy ligatures, two or three abreast, and in tightening them to employ only enough force to bring the intimal surfaces in contact, a force not sufficient to rupture or injure the intimal coat. The gross and microscopic findings in the sections of arteries ligated in this manner have been repeatedly portrayed, but the illustrations submitted as proof are not convincing.

It is my opinion that the pressure necessary to bring about the complete closure of the aorta causes atrophy of the arterial wall under the band, and that union of the apposed surfaces thus deprived of their blood supply does not occur. To accord with this view, how is to be explained the formation of the fibrous cylinder which we occasionally find encompassed by the band, and how the probable error of other investigators who believe that intimal surfaces brought gently in contact by broad ligatures unite primarily and thus interrupt the blood stream?

The process of occlusion is, I believe, somewhat as follows: The death of the arterial wall having been brought about by the pressure of the band, a gradual substitution or organization of the necrotic tissue takes place, the new blood vessels penetrating it from both ends. The absorption of the lifeless wall proceeds coordinately with its vascularization or organization.

If the band has been rolled so tightly as to occlude the lumen, the arterial wall is deeply puckered or plicated. If after a month or two the aorta is cut open at this point, the folds of the arterial wall may still be seen. They will not be adherent to each other, but atrophied possibly to the thinness of writing or tissue paper. The attenuated wall can still be completely unfolded. In some cases it is found to be abnormally thick and a very fine lumen to have been established (Fig. 13). In this event there are no folds, the thick wall consisting altogether of new tissue, and the old wall with its plication having been absorbed. Consequent upon the atrophy of the arterial wall the tension under the band is eliminated, capillaries sprout into the necrotic part, which thus becomes replaced by new connective tissue. A tiny blood stream may make its way under the band, and an endothelial lining for the new wall develop.

More often we find in the completely or almost completely occluded cases that a solid, fibrous, cylindrical cord completely fills the space within the band and replaces the original wall of the artery.

Although we are unable to share the opinion of other investigators that the uninjured intimal surfaces of large arteries adhere to each other when brought in contact, the advice to bring broad surfaces in apposition by several contiguous, coarse ligatures is good. We have found that the finer the ligature the quicker it cuts through the artery, very fine silk cutting through in a day or two. Narrow tape, constricting the artery in spiral or cuff-like form, has about the same effect upon the arterial wall as the metal band. Under three or four coarse ligatures, drawn tightly enough to occlude the lumen but not so tightly as to injure the intima, the arterial wall would behave presumably as it does under the tape or band, and occlusion take place in the described manner. I should think, however, that it would be impossible in a given case to be sure that each one of three ligatures, for example, had occluded the artery without injury to the intima. Even with the metal band, which can be rolled with great precision and in perfect, cylindrical form, it is not possible to say that occlusion has no more than just been attained, although we can be certain that the intima has suffered no trauma. But with the ligature pressure cannot be exerted in such fine and exact gradations; there is also the complication of the knot and its particular pressure.

The surgeon's conceptions as to the finer processes concerned in the occlusion of arteries after ligature are based largely upon studies undertaken before the days of perfected asepsis, in the days when thrombus formation almost invariably complicated the picture.

SUMMARY.

1. A partially occluded artery may dilate distal to the site of constriction.

2. The dilation is circumscribed.

3. When the constriction has been either slight in amount or complete, dilation has not been observed.

4. The dilation was greatest when the lumen of the artery (the aorta) was reduced to one-third or perhaps one-fourth of its original size (Figs. 8 and 11).

5. Dilation or aneurysm of the subclavian artery has been observed twenty-seven or more times in cases of cervical rib.

6. The dilation of the subclavian is circumscribed, is distal to the point of constriction, and strikingly resembles the dilation which we have produced experimentally.

7. The genesis of the experimental dilation and of the subclavian dilation occurring with cervical rib is probably the same.

8. When the lumen of the aorta is considerably constricted the systolic pressure may be permanently so lowered and the diastolic pressure so increased that the pulse pressure is greatly diminished.[3]

9. The experimentally produced dilations and the aneurysms of the subclavian artery in cases of cervical rib are probably not due to vasomotor paralysis, trauma, or sudden variations in blood pressure.

10. The abnormal, whirlpool-like play of the blood in the relatively dead pocket just below the site of the constriction, and the lowered pulse pressure may be the chief factors concerned in the production of the dilations.

11. Intimal surfaces brought, however gently, in contact by bands or ligatures do not, in our experience, unite by first intention, for

[3] See Reid, M. R., *J. Exp. Med.*, 1916, xxiv, 287.

the force necessary to occlude the artery is sufficient to cause necrosis of the arterial wall.

12. Bands, rolled ever so tightly, do not rupture the intima.

13. The death of the arterial wall having been brought about by the pressure of the band, a gradual substitution of the necrotic tissue takes place, the new vessels penetrating it from both ends. It is, I believe, in this manner that an artery becomes occluded, and it is thus that a fibrous cord forms within the constricting band.

TABLE I.

Cases of Dilation of the Subclavian Artery with Cervical Rib.

Surgeon.	Publication.	Note on dilation of subclavian artery.	Remarks.
Mayo, H.	*London Med. and Phys. J.*, 1831, N. S., xi, 40.	Right subclavian artery flattened. Unusual width.	
1. Adams, R.	*Dublin J. Med. Sc.*, 1836, xvi, 494.	Ovoid dilation of 3rd portion, left subclavian, about size of pigeon's egg. Postmortem confirmation.	Radial pulses equal.
2. Warren, J. M.	*Am. J. Med. Sc.*, 1849, xvii, 13.	Aneurysmal tumor, left subclavian, about size of pigeon's egg.	Dec. 24, 1847. Ligation of 3rd portion. Ligature came away on 96th day.
3. Coote, H.	Lancet, 1861, i, 360.	Pulsating swelling of left subclavian, about size of walnut.	Part of cervical rib resected. Radial pulse hardly palpable before operation.
4. Poland, A.	*Med.-Chir. Tr.*, 1869, lii, 277.	Right subclavian—"Circumscribed dilatation of entire circumference."	"Digital compression on cardiac side of tumor for 96 hours." Cure of aneurysm.
5. Baum, W.	*Deutsch. Chir.*, 1880, Liefg. 34, 25. (Fischer, G.)	"Left subclavian aneurysm."	Ice, elevation of arm, compression. Aneurysm cured. Pulse absent in radial and ulnar, but present in brachial.
6. Gould, A. P.	*Tr. Clin. Soc.*, 1884, xvii, 95.	"Right subclavian artery projected even beyond clavicle as tense pulsating cylinder."	No operation. Progressive obliterative arteritis. Dilation uninfluenced.
7. Morris, W. C.	*Lancet*, 1885, ii, 152.	Fusiform aneurysm of right subclavian, $\frac{3}{4}$ in. in width.	Operation. Central ligation of 3rd portion. Radial pulse before operation weak—absent after operation—but reappeared in 1 month or less.

* Karg.	*Beitr. klin. Chir.*, 1895, xiv, 215. (Ehrich, E.)	Aneurysm of left subclavian appeared after removal of the cervical rib.	Artery compressed between 2 cervical ribs. Both ribs removed. Aneurysm cured by compression.
8. Stifler.	*Münch. med. Woch.*, 1896, xliii, 544.	Pulsating tumor of left subclavian. Sudden appearance.	Blood pressure, left 120; right 140. No operation.
9. Cohen, J.	Inaug. Diss., Göttingen, 1898.	"Left subclavian aneurysm size of plover's egg."†	"Weaker radial pulse." Fusiform dilation.—"7 cm. long and 1½ fingers thick." Aneurysm excised.
10. Grisson.	*Fortschr. Geb. Röntgenstrahlen*, 1898–99, ii, 103.	Right subclavian, "pulsating cord, thickness of little finger."	Pulseless axillary, brachial (hard cord), and radial. Aneurysm cured by suspension, Priesnitz bandage, etc.
11. Winkler, C.	*Nederl. Tyschr. Geneesk.*, 1904, xl, 589; *Münch. med. Woch.*, 1904, li, 2111.	Left subclavian—"Locally aneurysmally dilated."	Cyanosis of left hand.
12. Murphy, J. B.	*Ann. Surg.*, 1905, xli, 399.	Left subclavian "flattened and circular."	Brachial and radial pulses normal with arm extended. Operation. Dilation questioned by author in 1905, but called abnormal dilation in 1906.
? Murphy, J. B.	*Surg. Gynec. and Obst.*, 1906, iii, 514.	Right subclavian flattened out on surface of rib.	(Circulation?) Dilation questioned by author. (Exophthalmic goiter.)

TABLE I.—*Continued.*

Surgeon.	Publication.	Note on dilation of subclavian artery.	Remarks.
13. Conner, L.	*Med. Rec.*, 1906, lxix, 775.	Left subclavian—"Broad pulsating mass suggestive of aneurysm."	No note as to circulation or treatment.
14. Keen, W. W.	*Am. J. Med. Sc.*, 1907, cxxxiii, 173.	Left subclavian dilated to nearly twice width of artery.	Gangrene. Operation. Cylindrical dilation to clavicle or beyond.
? Russel, C. K.	*Med. Rec.*, 1907, lxxi, 253.	*Autopsy Note.*—Right subclavian proximal to thyroid axis, large and soft; distal to this, contracted and cord-like. Left subclavian enlarged.	Gangrene. Amputation of index finger, and later of arm. Patient would not consent to operation on rib. Suicide.
? von Heinecke, W. H.	Inaug. Diss., Erlangen, 1907, 40. (Deitmar.)	Left subclavian "surprisingly broad." (Operative note.)	Left radial pulse weaker than right, not improved by resection of rib.
* Halsted, W. S.	Unpublished (1909).	Dilation of right subclavian developed after removal of cervical rib.	The aneurysm, size of plover's egg, seemed to involve the artery central to the site of constriction.
15. Hamann, C. A.	*Cleveland Med. J.*, 1910, ix, 453.	Right subclavian beyond rib enlarged and filled with clot.	Gangrene of fingers. Bursa $\frac{3}{4}$ in. in diameter between artery and rib. Resection of part of the rib.
16. Pringle, J. H.	*Edinburgh Med. J.*, 1911, vii, 253.	Left subclavian—fusiform aneurysm distal to rib—sacculated at its proximal part under outer edge of scalenus.	Aneurysm twice needled (before discovery of rib). Finally opened and rib removed. Radial pulse "feebler;" became fair after operation.

17. Miller, J. L.	*Am. J. Med. Sc.*, 1911, cxlii, 811.	Side not stated. Slight dilation above the notch in cervical rib.	Cyanosis of hand.
Galloway, J.	*Proc. Roy. Soc. Med.*, 1911–12, v, 112.	Left subclavian—"pulsating tumor."	Unoperated.
? Seymour, N. G.	*Am. J. Med. Sc.*, 1913. cxlvi, 396.	Right subclavian—small pulsating tumor, "simulating" aneurysm.	Female, 16 years. Radial pulse unaffected.
18. Law, A. A.	*J.-Lancet*, 1914, xxxiv, 333.	"Right subclavian dilated distal to rib." "Left subclavian flattened."	Gangrene of fingers. Resection of rib on both sides.
19. Potter, C.	*J. Missouri Med. Assn.*, 1914–15, xi, 74.	Left subclavian—"Pouch-like dilatation of the wall of the artery over groove in cervical rib."	Male, 17 years. Ulcers and gangrene of thumb and ring finger. Brachial and radial pulses absent.

In the unnumbered cases the dilation is questionable. In the cases marked with an asterisk the aneurysm appeared after removal of the rib.

† Possibly due to fracture of the clavicle.

EXPLANATION OF PLATES.

Plate 16.

DOG 7, SERIES I. THE FIGURES ARE ACTUAL SIZE.

Fig. 1. Aorta before the band was applied.
Fig. 2. Immediately after application of the band.
Fig. 3. 2 months thereafter.
Fig. 4. 6 months and 19 days thereafter.

Plate 17.

DOG 3, SERIES II. THE FIGURES ARE ACTUAL SIZE.

Fig. 5. Aorta before the band was applied.
Fig. 6. Immediately after application of the band.
Fig. 7. 97 days thereafter.
Fig. 8. 9 months thereafter. The outer of the two concentric circles indicates the circumference of the aorta at the site of the band before its application; the inner circle, the precise lumen of the aorta under the band when the dog was killed.

Plate 18.

DOG 15, SERIES II. THE FIGURES ARE ACTUAL SIZE.

Fig. 9. Aorta before the band was applied.
Fig. 10. Immediately after application of the band.
Fig. 11. 160 days thereafter. The outer of the two concentric circles indicates the circumference of the aorta at the site of the band before its application; the inner circle, the precise lumen of the aorta under the band when the dog was killed.

Plate 19.

SECTION OF HUMAN INTERNAL CAROTID.

Fig. 12. Section of human internal carotid, showing the process of obliteration after ligation of the common carotid. When removed at a subsequent operation this artery was found pulseless and almost empty notwithstanding the size of its lumen. $\times$ 25.

DOG 9, SERIES II. THE FIGURE IS ACTUAL SIZE.

Fig. 13. The band was rolled so tight as to shut off the palpable thrill but not the pulse. When killed after 6 months and 8 days the lumen at the lower edge of the band was 1.2 mm. in diameter. A new wall had been formed under the band, thick throughout, but particularly so at the lower and upper borders of the band. It is probable that, in this case, a fibrous cord ultimately would have formed.

PARTIAL OCCLUSION OF THE AORTA WITH THE METALLIC BAND. OBSERVATIONS ON BLOOD PRESSURES AND CHANGES IN THE ARTERIAL WALLS.*

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Plates 20 to 26.

(Received for publication, June 27, 1916.)

In all except one of the aortic experiments of Dr. Halsted and myself the constricting aluminum band was applied to the abdominal aorta below its inferior mesenteric branch. At the time of our final observations on these animals records were made of the blood pressures in the femoral and carotid arteries. Obviously, in order to draw any conclusions as to the effect of the band on the blood pressure below the site of the constriction, the normal relation between the pressures in these two vessels must be known.

In a series of experiments performed by Dr. Dawson on dogs, it was learned that the pulse pressure in the femoral artery is normally about twice as high as in the carotid. The femoral systolic pressure is higher and the diastolic pressure lower than the corresponding pressures in the carotid artery (Fig. 1).

After partial occlusion of the aorta the systolic pressure in the femoral is markedly lowered. This lowering of the systolic pressure is due mainly to a fall in the pulse pressure, for the diastolic pressure remains almost stationary, or may be actually increased. In the cases of most marked dilation the femoral pulse pressure was only about one-half the carotid pulse pressure, while the femoral diastolic was actually greater than the carotid diastolic pressure (Fig. 2).

*This communication is to supplement Dr. Halsted's paper on Circumscribed Dilation of an Artery Immediately Distal to a Partially Occluding Band, *J. Exp. Med.*, 1916, xxiv, 271.

During the 1st hour after the application of a moderately tight band the femoral pressures undergo marked changes. At first the systolic and diastolic pressures are both lowered. In a few minutes the diastolic pressure may become even greater than before the application of the band, while the systolic is still subnormal (Fig. 3).

After complete occlusion of the aorta the normal blood pressure relation between the femoral and carotid arteries may, ultimately, in some instances, be reestablished.

Gross Effect of the Band on the Vessel Wall.—In some cases in which the band has been loosely applied, only slight gross alteration in the wall of the vessel under the band is found, even after 6 months. On removal of the band the plications of the wall can be unfolded, and the intima presents a normal looking, smooth surface (Fig. 4).

In the majority of cases there occurs an atrophy or necrosis of the vessel wall included in the band. In some cases the band had made its way through the atrophic wall into the lumen of the vessel (Figs. 5, 6, and 7); in these a new wall had formed outside the band. In no instance was there leakage of blood. On splitting open the artery for examination the band is seen, more or less distinctly, shimmering through the atrophic arterial wall. This wall may be attenuated to a veil-like thinness or, as described, may, in places, have entirely disappeared, disclosing portions of the band. The upper edge of the band, at the posterior surface of the arterial wall, is almost invariably the first part to be exposed (Figs. 5 and 6).

Histologic Changes.—For a short distance below the site of the band there is usually a definite atrophy of the elastic and muscular tissues. In Case 7, Series I, (Fig. 8, *c*), there was almost a complete break in the elastic tissue below the edge of the band. The connective tissue throughout the wall of the artery seemed to be little affected in amount in the dilated portion of the vessel.

At the site of the band the new wall that forms over it is composed mainly of fibrous tissue (Figs. 7 and 9). Thus far, none of our cases have shown regeneration of the elastic tissue in this new wall.

In the fibrous cord (Figs. 10, 11, and 12) which occasionally forms under the tightly rolled band no remains of the vessel wall have been found. We think it probable that the original arterial wall undergoes complete atrophy and absorption in these cases, and that

the cylindrical cord found under the band consists of new tissue which, growing in from above and below, replaces the old. This cylindrical fibrous cord may be highly vascularized (Fig. 12). We have found no evidence of union between the apposed intimal surfaces.

EXPLANATION OF PLATES.

Plate 20.

Fig. 1. The normal relation between the systolic and diastolic end pressures in the femoral and carotid arteries of dogs.

Fig. 2. Systolic and diastolic end pressures in Dog 7, Series I, 6 months and 19 days after the band was applied. Good dilation.

Fig. 3. Systolic and diastolic end pressures in the femoral artery during the first 35 minutes after a tight band was placed around the aorta.

Plate 21.

Fig. 4. Dog 9, Series I. The band was rolled to the early stage of palpable thrill. The band in 6 months has produced only slight thinning of the vessel wall.

Fig. 5. Dog 7, Series II. Marked constriction. The thrill was not obliterated. The band had, in 6 months, cut entirely through at its upper edge, posteriorly. At the lower edge of the band there is still a remnant of the atrophied wall (compare Fig. 7).

Fig. 6. Dog 0, Series II. The band was tightly rolled but not totally occluding. At autopsy, 5½ months later, the lumen under the band was about 3 mm. in diameter. The aortic wall was so thin that the band shimmered through it everywhere, and had cut through at several places along the upper and lower edges. A new arterial wall, almost complete, had formed outside the band.

Plate 22.

Fig. 7. Dog 7, Series II. Longitudinal section through the arterial walls, old and new (compare Fig. 5). Weigert's elastic tissue stain. *a* is the site of the band; *b*, elastic tissue of the aortic wall proximal to the band; *c*, elastic tissue distal to the band; *e*, the new wall which had formed outside the band where it had cut through, posteriorly. Between *a* and the lumen of the vessel the original wall is greatly thickened.

Fig. 8. Dog 7, Series I. The band was tightened until the thrill disappeared; the pulse was not obliterated. Killed after 6 months and 19 days. Longitudinal section through the arterial wall at the site of the band. Weigert's elastic tissue stain. In this case there was marked dilation below the band. *a* is the

site of the band; *b*, the aortic wall proximal to the band; *c*, the aortic wall distal to the band, showing definite atrophy and a break in the elastic tissue; *e*, the new wall that had formed outside the band; *x* to *x*, the segment of the vessel wall that had been included by the band. In this segment there is marked atrophy of the elastic tissue, particularly at the edges of the band.

PLATE 23.

FIG. 9. Dog 17, Series I. The band was tightened to the vanishing side of the thrill stage. Longitudinal section through the arterial wall at the site of the band. Weigert's elastic tissue stain. Under the band the elastic tissue is rarified. Below the band, where there was a moderate amount of dilation, atrophic changes are noted. *a* is the slit occupied by the band; *b*, the vessel wall proximal to the band; *c*, the vessel wall distal to the band.

PLATE 24.

FIG. 10. Dog 2, Series I. The band was tightened until the pulse had just disappeared. When examined after 7 months, a solid fibrous, cylindrical cord was found under the band.

PLATE 25.

FIG. 11. Dog 2, Series I. Longitudinal section through the fibrous cord shown in Fig. 10. Weigert's elastic tissue stain. *a* is the site of the band, under it the elastic tissue has vanished; *b*, the vessel wall proximal to the band; *c*, the vessel wall distal to the band, showing definite atrophy of the elastic tissue; *d* to *d*,' the fibrous cord; *e*, fibrous tissue that formed about the band. There is almost complete disappearance of the elastic tissue at the site of the band.

PLATE 26.

FIG. 12. Dog 2, Series I. Transverse section of the fibrous cord through ***d***, Fig. 11. Hematoxylin and eosin.

DISTORTION OF THE MALARIAL PARASITE. AN INTERPRETATION OF "PLASMODIUM TENUE" (STEPHENS.)*

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PLATES 27 TO 35.

(Received for publication, April 7, 1916.)

Types of Malarial Parasites.

Practically all authorities agree that there are at least three distinct species of malarial parasites: the tertian parasite, the quartan parasite, and the æstivo-autumnal parasite. This classification is based not only on the morphological and biological characteristics, but also on the clinical manifestations. A few observers have divided the æstivo-autumnal fevers into two groups, one causing quotidian fever, the other malignant tertian. But no convincing morphological basis has been established for making this distinction, and Marchiafava and Bignami,[1] who have so divided the æstivo-autumnal fevers, admit that they are "to be distinguished from each other not so much by their morphological characters as by the mode of their pathogenic action upon man."

Malarial Parasites Recently Described as New Types.—In 1914 papers were published describing certain malarial parasites which were believed by the authors to be new varieties. Emin[2] describes a parasite which he has named *Plasmodium vivax, variete minuta*, but it is not my purpose to discuss Emin's parasite in this paper.

Stephens[3] named the parasite described by him *Plasmodium tenue*. A little

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth Century Practice, New York, 1900, xix, 29.

[2] Emin, A., Une variété nouvelle du parasite de Laveran, *Bull. Soc. path. exot.*, 1914, vii, 385.

[3] Stephens, J. W. W., A New Malarial Parasite of Man, *Ann. Trop. Med. and Parasit.*, 1914, viii, 119.

later he[4] published another article apparently referring to parasites similar to those described by him under that name.

In order to make a correct interpretation of the parasites described by Stephens, there are three facts that the observer must bear in mind: (1) that the malarial parasite is attached to corpuscular mounds on the external surface of the red corpuscle; (2) that more than one parasite may be attached to a single corpuscular mound; (3) that the nuclei of young parasites contain a large amount of chromatin.

The Extracellular Relation of the Malarial Parasite to the Red Corpuscle.[5]

All malarial parasites are extracellular throughout their entire life cycle. The following figures show the external relation of the parasite to the red corpuscle. (*a*) Parasites attached to peripheral corpuscular mounds (Figs. 3, 4, 5, 7, 11, 15 to 23, 42, 51, 56, 110, to 112, 168, 237, 238, 242, and 253); (*b*) parasites on the periphery of the infected red corpuscles (Figs. 6, 9, 10, 21 to 33, 36 to 47, 49, 50, 54, 57 to 62, 66, 79, and 239 to 248; (*c*) parasites attached to the upper or under surface of red corpuscles with nuclei or portions of their cytoplasm extending beyond the periphery of the corpuscles to which they are attached (Figs. 1, 2, 48, 52, 53, 55, 63 to 65, 67, 74, 76, 84 to 87, 91, 161 at o, 164, 203 at o, 249, 250, 254, and 264); and (*d*) red corpuscles with Schüffner's granules but no parasites (Figs. 68, 69 at o, 70, and 71).

(*a*) Malarial parasites capture and attach themselves to the outer surface of the red corpuscles by means of pseudopodia arising from the cytoplasm of the parasites. They maintain their position on the external surface of the corpuscles by encircling one or more mounds of hemoglobin substance squeezed up by the parasites for the purpose of attachment. This interpretation of the relation of the para-

[4] Stephens, On the Peculiar Morphological Appearances of a Malarial Parasite, *Ann. Trop. Med. and Parasit.*, 1915, ix, 169.

[5] The author has shown in previous publications that the malarial parasite is extracellular. See Rowley-Lawson, M., *Arch. Int.* Med., 1912, ix, 420; *J. Exp. Med.*, 1913, xvii, 324; 1914, xix, 450; 1915, xxi, 584.

sites to the corpuscles is verified by the mounds being encircled by the parasites at the periphery of the corpuscles. These parasites admit of no other interpretation than that they are on the outer surface of the corpuscles, and it seems unreasonable to suppose that they would use this method of attachment only when situated at the periphery of the corpuscles. Moreover, it must be remembered that this method of attachment gives the only rational explanation of the ring-forms.

(*b*) Parasites resting on the periphery of the red corpuscles with their pseudopodia encircling peripheral or surface mounds.

(*c*) Parasites with nuclei or a portion of their cytoplasm extending beyond the periphery of the corpuscles to which they are attached, show these conditions so clearly that even the most confirmed believer in the intracellular theory must acknowledge that they are on the external surface of the corpuscles. It is the nucleus of the parasite that is most commonly seen projecting beyond the periphery of the infected corpuscle. This occurs when the parasite is attached to a mound at or near the periphery of the corpuscle (Figs. 1 to 3, 7, 18, 19, 50, 53, 67, 84, 91, 203 at o, and 237).

Parasites partly off corpuscles or parasites attached to the surface of corpuscles with their bodies resting on the periphery, are most commonly found at the time of a parasitic migration when the parasite is either abandoning the dehemoglobinized corpuscle or attaching itself to a fresh red corpuscle (with normal color).

Occasionally a parasite may have its nucleus or a portion of its cytoplasm drawn off the infected corpuscle by technique without appreciable injury to the corpuscle (Figs. 46 to 49, 51 to 53, 55, 56, 58, 62, 74, 76, 85 to 87, 161, 164, 246, 249, 253, 254, and 264).

(*d*) Red corpuscles showing Schüffner's granules but having no parasites attached to them may occasionally be found in malarial infections (Figs. 68 to 71).

Schüffner's granules differ in morphology and staining reaction from Maurer's dots and rings, from the blue stippling of red corpuscles, and from the granular corpuscular skeletons. The following figures show that the granulations of these corpuscles without parasites are not to be confounded with other stippling or granula-

tion: (*a*) a red corpuscle with Schüffner's granules and no parasite next to a red corpuscle with Schüffner's granules and a parasite (Fig. 71); (*b*) a red corpuscle with Schüffner's granules and no parasite next to a red corpuscle with blue stippling (Fig. 69); (*c*) a red corpuscle with Schüffner's granules and a parasite next to a corpuscular skeleton or dehemoglobinized red corpuscle (Fig. 73); (*d*) three red corpuscles showing Maurer's dots and rings (Figs. 78 to 80). Two of these corpuscles are infected by æstivo-autumnal parasites (Figs. 78 and 79), and one is infected by two young quartan parasites (Fig. 80).

All observers at present agree that Schüffner's granules are always associated with corpuscles which harbor malarial parasites; and that the granules are the effect of the actual presence of the parasites; that is, the parasites must have been attached to these corpuscles before the granulation could have occurred.

It might be suggested that these granules resulted from something other than the actual presence of an invading parasite; but such an hypothesis is highly improbable. The natural supposition would be that the parasites had been attached to the corpuscles long enough to cause the granulation, and then, for some reason had abandoned them. If this is true, the invading parasites must have been attached to the external surface in order to have escaped without visible injury to the contour of the infected corpuscles.

Ever since Marchiafava and Celli[6] decided that malarial parasites were intracellular because they never observed the pseudopodia beyond the periphery of the infected corpuscles, few observers have ventured to dispute their theory. Mannaberg[7] did not consider the young forms to be intracellular, and in referring to Marchiafava and Celli's findings, he states: "The criticisms brought forward by Marchiafava and Celli are not a confirmation of their view, for the adhesiveness of the parasites would prevent their ever, even during ameboid movement, becoming free from the blood corpuscles."

Schaudinn[8] pictures an adult tertian parasite with a portion of its cytoplasm

[6] Marchiafava and Celli, quoted from Marchiafava and Bignami, Malaria, in Stedman, T. L., Twentieth Century Practice, New York, 1900, xix, 29.

[7] Mannaberg, J., Malarial Parasites, London, 1894, 276.

[8] Schaudinn, F., Studien über krankheitserregende Protozoen. II. ***Plasmodium vivax*** (Grassi & Feletti), der Erreger des Tertianfiebers beim Menschen, ***Arb. k. Gsndhtsamte.***, 1902, xix, 169, and Plate V, Fig. 86.

extending beyond the periphery of the infected corpuscle. Gautier[9] depicts the appearance of the ring projecting beyond the cell. And Ewing[10] states: "It is generally accepted that the tertian parasite lies within the red cell yet, in many tertian cases the body and especially the nucleus of the parasite appear to project beyond the border of the cell, even more distinctly than in the case of the æstivo-autumnal ring." Loeffler[11] shows in a colored plate three young æstivo-autumnal parasites, the nuclei of which project beyond the periphery of the infected corpuscle.

Multiple Infection of Corpuscular Mounds.

The second fact to bear in mind in order to interpret successfully Stephens' *Plasmodium tenue* is that more than one parasite may occupy one corpuscular mound.

When more than one parasite is attached to one corpuscular mound, and the cytoplasm of the last parasite to attach itself is superimposed over that of the other parasite or parasites, one may get the impression of a single ring with several masses of chromatin. This is especially true in the case of the young parasite where practically all the cytoplasm is required to enable it to encircle a mound (Figs. 2, 8, 14, 119 at o, 125 at o, and 143 at o).

This occurrence has been variously interpreted by many observers. Emin,[12] in the description of his new parasite, gives examples of two and three young parasites occupying one corpuscular mound and interprets the condition as that of a precocious division of the chromatin.

Many of the pictures of multiple infection of corpuscular mounds were selected so that the parasites occupying that position could readily be recognized as individuals (Figs. 7, 10 to 13, 22, 34, 35, and 113), and not be interpreted as conjugating parasites or single parasites with more than one chromatin mass.

[9] Gautier, quoted from Ewing, J., Malarial Parasitology, *J. Exp. Med.*, 1900–01, v, 474.

[10] Ewing, Malarial Parasitology, *J. Exp. Med.*, 1900–01, v, 474.

[11] Loeffler, F., Malarial Diseases, in Modern Clinical Medicine, New York, 1910, Colored Plate H.

[12] Emin, Une variété nouvelle du parasite de Laveran, *Bull. Soc. path. exot.*, 1914, vii, Plate IV, Figs. 3 to 5.

The Large Amount of Chromatin in the Nuclei of Young Parasites.

The third fact to remember is the large amount of chromatin which the nuclei of young parasites contain. I think that this fact is readily recognized by most observers. To illustrate this I have selected pictures of young æstivo-autumnal parasites attached to red corpuscles with their nuclei drawn out by technique beyond the periphery of the corpuscles to which the parasites are attached (Figs. 85 to 87, and 264).

These examples serve to illustrate not only the large amount of chromatin in the nuclei, but prove the external relation of these parasites to the red corpuscles; for were they within the substance of the corpuscles, such a condition as that pictured would obviously be impossible.

The entire set of nuclei from a segmenting tertian organism forcibly removed from the parasites with strings of chromatin drawn out by technique (Fig. 83) also serves to illustrate the enormous amount of chromatin contained in the nuclei of young parasites.

Parasites Distorted by Technique (Plasmodium tenue). (Figs. 92 to 269.)

Several observers have written about Stephens' *Plasmodium tenue* without giving a rational explanation of the peculiar appearances presented by the parasites illustrated, but criticizing him for drawing his conclusions from a single blood film. I shall now give what I believe to be the true interpretation of the forms that he shows.

Stephens drew his conclusions wholly from the peculiar morphological appearances of the parasites which he has demonstrated in both of his articles. I judge that he believes the malarial parasite to be intracellular. He states in his second article: "forms are found in which chromatin alone without any protoplasm occurs in the red cell;" and he also suggests that the parasites cannot be distorted by technique without apparent distortion of the infected red corpuscle. In the same article he writes: "I would point out that the blood cells present no evidence whatsoever of stretching or distortion," and if the parasites he pictures were within the substance of the red corpuscle they could not be distorted by technique and the corpuscle retain its normal contour.

The unusual appearances presented by the parasites he pictures are due entirely to technique. They are young, unpigmented parasites attached to the external surface of the red corpuscles and drawn out of shape during the process of spreading the blood film. I have seen these forms many times.

A careful study of the parasites resting on the periphery of the corpuscles shown in the accompanying plates will suggest the ease with which they could be distorted, and it is surprising that we so often get normal-appearing parasites with our crude methods of spreading films. Possibly the corpuscular mounds, to a certain extent, protect the parasites from distortion; but while it is comparatively easy to distort parasites, they are not easily dislodged.

Distorted parasites are produced accidentally; that is, you may get them even with the most careful technique. Violent technique usually distorts the corpuscles as well as the parasites. With a heavy infection, the blood may not spread freely and may stick slightly when the cover-slips are drawn apart, thus distorting the parasites. Occasionally parasites are distorted if uneven pressure is used in spreading the blood on a slide. They are most commonly found in that part of the specimen where the film is unevenly spread, and along the edges of films made on slides and cover-slips. They may be found where the film is thickly spread as well as where it is thin. If thoroughly examined, almost any specimen of malarial infection containing young parasites, will show a few distorted forms. It is rare to find a specimen in which the greater number of parasites is markedly distorted, but I have seen a few specimens where the normal parasite was the exception. Stephens states in both articles that ring-forms were present in his specimens. "Forms resembling 'rings' do occur," and "it is possible to trace a transition from normal ring-forms," etc.

Types of Distorted Parasites.—In general three types of distorted parasites may be recognized: (*a*) parasites with nuclei distorted and cytoplasm unaltered or but slightly distorted (Figs. 105 at o, 117 at o, 125 at x, 126 to 129, 131 at x, 135, 138, 139, 140 at x, 141, 145 at x, 147, 149, 153, 154 at x, 169, 171, 172, 191, and 192); (*b*) parasites with cytoplasm distorted and nuclei intact or but little disturbed (Figs. 98 at x, 102 at x, 130, 156, 158, 160, 161, 164, 165, 178, 183,

193, 194, 195, 196, 197, 207, 210, 211, 226 at x, and 232 to 236); (*c*) parasites with nuclei and cytoplasm markedly or slightly distorted (Figs. 131 to 133, 136, 137, 140, 142, 144 to 146, 148, 150 to 152, 154, 155, 157, 159, 162, 166 to 168, 170, 173 to 177, 179 to 181, 184 to 190, 199 to 204, 206, 208, and 212 to 231).

Evidence That the Appearance Presented by the Parasites Described by Stephens Is Due to Technique.—If one examines a specimen showing a rich infection and containing many of the forms which I have described as distorted parasites, one will find certain parts of the film where the parasites are drawn out, in one general direction, while the infected corpuscles, although they may be flattened out, retain their normal contour (Figs. 102, 148, 150 to 152, and 156 to 160). And groups of parasites will be found with their nuclei similarly distorted (Figs. 147, 149, 153, and 167).

These facts should suggest technique as the cause, provided that one is willing to concede that the parasites are attached to the external surface of the red corpuscles. No other explanation seems possible, since bizarre parasites could not be limited to certain parts of the blood film.

When two or more parasites attached to one corpuscle are seen stretched across the corpuscle, in one direction (Figs. 102, 150, 152, 155, 156, 158, 165, 166, 171, 172, 177 to 180, 188, 189, 197, 222, and 227 to 230), technique should suggest itself as the cause.[13]

Distortion of Parasites Occupying One Corpuscular Mound.—Instances of two or more parasites occupying one corpuscular mound, when the parasites are distorted by technique, present appearances such as may be seen in Figs. 117 at o, 128 at x, 129 to 131, 138, 139, 142, 165, 166, 169, 170, 180, 195, 198, 206, 207, 209 to 212, 222, and 226 at x.[14]

Fusion of Chromatin.—The nuclei of two or more parasites may be so fused together by technique as to give the appearance of a single mass or string of chromatin. This may be seen in Figs. 105

[13] Stephens shows this condition in Figs. 19, 26, 27, 30, 31, and 32, of the colored plate illustrating his first article.

[14] This is also shown in Figs. 2 to 18, 20, 23 to 25, 28 to 31, and 33 to 35 of the colored plate illustrating Stephens' first article.

at o, 117 at o, 127 at o, 129, 139, 142, 166, 169 at o, 192, 195, 198, 206, 209, 210, and 212.[15]

How to Obtain Distorted Parasites So That They May Be Identified as Such.—Take several films from one large drop of blood, using varying technique in preparing them. It is more satisfactory to work this out with a heavy infection, showing several parasites in one field. One may have to make films from many specimens to obtain distorted parasites, or one may secure them in the first attempt. Distorted parasites in one or two of a set of films and none in the others indicate that the distortion is due to technique.

I have several times secured distorted parasites in this manner, accidentally. I frequently take four cover-slips from one drop of blood in order to study various phases in the life of the parasite. Occasionally one of the specimens contains certain distorted parasites, while in the other specimens the parasites are normal. Without other evidence, this would be proof that these forms are due to technique.

Plasmodium tenue.—This is an appropriate description of the drawn out, flattened parasite. As one would expect, it shows but little density compared with the normal parasite; the bulk of cytoplasm varies with the age of the parasite. Stephens writes: "The parasite has but little bulk, or density."

Many of the distorted æstivo-autumnal parasites in Figs. 115, 117 and 118 to 146 show but little cytoplasm compared with the amount of chromatin. These parasites are very young. (The normal parasites from the same film may be seen attached to corpuscles in Figs. 1 to 8, and free in Figs. 118 to 124.) The specimen containing these parasites was taken 15 minutes after a paroxysm, and contained, besides the parasites attached to the red corpuscles, segmenting bodies, free segments, phagocytes with inclusions of segmenting bodies and pigment masses, as well as free pigment masses. Two cover-slips were taken from one drop of blood; one contained the distorted forms shown, the other normal parasites.

The distorted æstivo-autumnal parasites shown in Figs. 168 to

[15] This may also be seen in Figs. 2 to 4, 7, 12, and 16 of the colored plate accompanying Stephens' first article.

181 were taken from a specimen of blood secured 1 hour after a paroxysm. Figs. 9 to 14 show normal parasites attached to corpuscles, which were taken from the same specimen. The distorted æstivo-autumnal parasites shown in Figs. 147 to 167 were from the same case as the normal parasites in Figs. 15 to 34. The distorted quartan parasites in Figs. 183 to 192 correspond in age to the quartan parasites in Fig. 80.

Adult as well as young parasites may be flattened and drawn across the corpuscle by technique. The tertian forms (Figs. 232 to 236) were from one film and were distorted in the same general direction. This type of distorted parasite corresponds to the so called quartan "*bandform*" parasite pictured by Ruge,[16] Kolle and von Wassermann,[17] Ziemann,[18] Loeffler,[19] von Wasielewski,[20] and others.

Red Corpuscles to Which Distorted Parasites Are Attached.—Parasites may be more or less distorted without apparent flattening of the infected corpuscle, or change in its circular contour; but this is not the rule. Red corpuscles to which these parasites are attached, usually appear to be ironed out, and the adjacent uninfected corpuscles may give the same appearance. Practically all the infected red corpuscles shown in both of Stephens' articles have a slightly irregular outline and appear to be flattened out. This makes it difficult to ascertain whether the distorted parasites enlarged the infected corpuscles or otherwise, and it explains why Stephens found it difficult to determine whether the *Plasmodium tenue* enlarged the corpuscle or not. He writes: "I am not sure whether this parasite enlarges the cell, as, although in some cases I found infected cells larger than non-infected ones in their vicinity, in other cases the reverse held good."

[16] Ruge, R., Einführung in das Studium der Malariakrankheiten mit besonderer Brücksichtigung der Technik, Jena, 1901, Plate I, Fig. 16.

[17] Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Atlas, Jena, 1902, Plate III, Fig. 65.

[18] Ziemann, H., Malaria, in Mense's Handbuch der Tropenkrankheiten, Leipzig, 1906, iii, 269; Plate IX, Figs. 39 and 41.

[19] Loeffler, Malarial Diseases, in Modern Clinical Medicine, New York, 1910, Plate I (C), Figs. 2 and 3.

[20] von Wasielewski, T., Plasmodiden, in Rubner, Gruber, and Ficker's Handbuch der Hygiene, 3te Abt., Leipzig, 1913, iii, Plate 26, Fig. A, 4, 6.

Stephens' Forms in Which Chromatin Particles or Strands without Protoplasm Occur in the Red Cell.—Stephens refers to this condition in his second article. His views as to the probable nature of the forms he describes are as follows:

"(1) That they are a new species of parasite

"(2) That they are degenerative, i.e., formed in the body under unknown conditions, and so perhaps analogous to the so-called quinine forms of parasites.

"(3) That they are artificial, i.e., formed outside the body under unknown conditions."

And he goes on to say: "The fact that among the parasites figured, forms are found in which chromatin alone without any protoplasm occurs in the red cell is, I think, in favor of, but not decisive for, one of the latter two views rather than the first." He states that forms consisting of protoplasm alone, without chromatin, were not seen.

The chromatin masses without any cytoplasm which he describes as being present, are, I believe, stained remnants of parasites whose cytoplasm has faded.

Parasites Whose Chromatin or Cytoplasm Has Faded or Failed to Stain.—There are two general types of partially stained parasites which may be described as follows: (*a*) parasites whose chromatin remains stained, but whose cytoplasm has faded or failed to stain (Figs. 88 to 90, 101, 105 to 109, 111, 112, 114, 116, 266, 267, and 269); and (*b*) parasites whose cytoplasm remains stained, but whose chromatin has faded or failed to stain (Figs. 92 to 97, 100 at o, 102 at o, 103, 104, and 268).

The examples of *a* and *b* shown herewith were taken from specimens containing successfully stained parasites. It is rare to find examples of both *a* and *b* in one film unless it is fading rapidly.

One may find parasites without nuclei on corpuscles to which are attached parasites with well stained nuclei (Figs. 92 to 97, and 100). That these nuclei might have been removed from the parasites by technique was considered; but as the gradual fading of the chromatin of various parasites could be followed, and a careful search failed to reveal free nuclei, the only reasonable conclusion to be reached was that the chromatin had faded. The gradual fading of cytoplasm may be followed in the same way.

Stained nuclei of parasites whose cytoplasm has faded are shown well in Figs. 88 to 90. Here the nuclei are successfully stained, as well as the crescents seen near them. Well stained young parasites were seen in the same film (Fig. 91).

Distorted chromatin of partially faded parasites may be seen in Figs. 105 to 109, 112, and 116, and chromatin which has not been distorted may be seen in Figs. 111 and 114.

If specimens showing the above conditions are restained, it is often possible to bring out the cytoplasm in connection with the chromatin, or the chromatin in connection with the cytoplasm.

Species of Parasite Described in Stephens' First Article.—The distorted parasites described by Stephens as *Plasmodium tenue* might be tertian, quartan, or æstivo-autumnal parasites; but judging from the delicate morphology of the parasites and the numerous instances of multiple infection of corpuscular mounds and of red corpuscles, I should judge them to be of the æstivo-autumnal type. In my experience the multiple infection of red corpuscles by young parasites is much more common in the æstivo-autumnal than in other malarial infections.

In the last paragraph of Stephens' second article, he refers to "further films from the case of *P. tenue*. Two films (March, 1914) showed quartan, and in one of the films a single pigmented (presumably simple tertian) parasite was found with the pigment in the form of rods, in an enlarged cell showing Schüffner's dots. Three films (June) showed quartan, and one (July), quartan." I should not say that this was evidence that the first specimen containing *Plasmodium tenue* forms was from a case of quartan infection.

The specimen containing the *Plasmodium tenue* forms described by Stephens in his first paper, was sent to him in the autumn of 1913, and if this case remained untreated or infected until March, 1914 (the first date stated above), one could not, after this lapse of time, be sure of dealing with the same infection unless one could eliminate the possibility of a reinfection by other species of malarial parasite. This would be especially true were the patient living in a malarial district.

The type of infecting parasite cannot always be designated by the occasional presence of Schüffner's granules or Maurer's rings in

the infected red corpuscle. The following figures are evidence of this: (*a*) a crescent attached to a red corpuscle showing Schüffner's granules (Fig. 77); (*b*) a quartan parasite attached to a red corpuscle showing Schüffner's granules (Fig. 81); (*c*) two young quartan parasites attached to a red corpuscle showing Maurer's rings and dots (Fig. 80). These are shown in connection with a crescent and an æstivo-autumnal ring-form parasite attached to red corpuscles showing Maurer's rings (Figs. 78 and 79). And one occasionally sees æstivo-autumnal ring-forms attached to red corpuscles showing Schüffner's granules.

Pigment of Malarial Parasites.—It should be mentioned here that too much importance must not be attached to the form which the pigment granules present in the parasites, for this pigment is subject to distortion, just as the parasites are, and it may be distorted without distortion of the parasites, just as the parasites may be distorted without distortion of the infected corpuscles. Much of the rod-shaped pigment seen owes its rod shape to technique.

Distorted Parasites Previously Described.

Previous to Stephens' two articles describing distorted parasites, several papers came out illustrating various types of parasites distorted by technique. In 1904 the author[21] published an article entitled, "Unusual Forms of Malarial Parasites," in which were pictured elongated forms of malarial parasites from æstivo-autumnal infections, wrongly interpreting these forms as stages in the life cycle of the crescent. In 1906 Ziemann[22] called attention to distorted forms of the æstivo-autumnal parasite. In 1908 Balfour and Wenyon[23] pictured distorted forms of the æstivo-autumnal parasites, interpreting them as ameboid forms. In 1911 in an article on the æstivo-autumnal parasite, the author[24] pictured various types of distorted parasites, stating that they were the result of technique.

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[21] Rowley, M. E., Some Unusual Forms of Malarial Parasites, *Bull. Johns Hopkins Hosp.*, 1904, xv, 22.

[22] Ziemann, Malaria, in Mense's Handbuch der Tropenkrankheiten, Leipzig, 1906, iii, 294.

[23] Balfour, A., and Wenyon, C. M., Sanitary Notes. Khartoum, *Third Rep. Wellcome Research Lab., Gordon Memorial College, Khartoum*, 1908, Colored Plate 7.

[24] Rowley-Lawson, M., The Æstivo-Autumnal Parasite: Its Sexual Cycle in the Circulating Blood of Man, with a Description of the Morphological and Biological Characteristics of the Parasite, *J. Exp. Med.*, 1911, xiii, 263.

In 1913 Sergent, Beguet, and Plantier[25] published an article in which attention is called to young æstivo-autumnal parasites with streaked arrangement of the chromatin. These forms are due to technique.

In 1914 Stephens published his article on distorted forms, "*P. tenue*," and this called out criticism and other illustrations of distorted parasites from Balfour and Wenyon[26] and Ziemann,[27] although these authors did not interpret the forms as due to technique. But in their last paper Balfour and Wenyon recognize that "it may be possible to find for these variations some mechanical explanation." In 1901 Ewing[28] mentions, in connection with the æstivo-autumnal ring-form parasite, that "occasionally such rings were found to have unfolded and to be stretched like a thread across the cell, the nuclei appearing at inconstant intervals." And in connection with the tertian ring-form parasite, he[29] writes: "Elongated forms of the young parasite are often seen in which the ring is absent and the nuclear body lies bare at one end." He undoubtedly refers here to parasites distorted by technique.

SUMMARY.

1. The correct interpretation of the form described by Stephens under the name of *Plasmodium tenue* is as follows: It is not a new species of parasite or an ameboid form, but a parasite attached to the external surface of red corpuscles and distorted by technique.

2. Evidence against a new species of parasite is: (*a*) they may be found in all known malarial infections and at any stage in the development of the parasite; and (*b*) they show evidence of distortion.

3. Evidence against ameboid forms and in favor of parasites distorted by technique is: (*a*) they may be found in but one of the two cover-slips prepared simultaneously; (*b*) they may be found in certain definite groups in various parts of a film which otherwise contains normal appearing parasites; (*c*) in rich infections, containing many disforted forms, they may be found in certain parts of a film, all distorted in one direction, the infected corpuscles and those adjacent to them showing no evidence of injury; and (*d*) two or more

[25] Sergent, Edw., Sergent, Et., Beguet, M., and Plantier, A., Observations microscopiques au cours d'un acces pernicieux paludées, *Bull. Soc. path. exot.*, 1913, vi, 615.

[26] Balfour and Wenyon, The So-Called *Plasmodium tenue* (Stephens), *J. Trop. Med.*, 1914, xvii, 353.

[27] Ziemann, Ueber eigenartige Malariaparasitenformen, *Centr. Bakteriol., 1te Abt., Orig.*, 1915, lxxvi, 384.

[28] Ewing, Malarial Parasitology, *J. Exp. Med.*, 1900–01, v, 446.

[29] Ewing, Malarial Parasitology, *J. Exp. Med.*, 1900–01, v, 436.

parasites attached to one red corpuscle may be seen to be distorted in one direction, or to have nuclei similarly distorted.

4. Three general types of distorted parasites may be recognized: (*a*) parasites with nuclei distorted and cytoplasm unaltered or but slightly distorted; (*b*) parasites with cytoplasm distorted and nuclei intact or but little disturbed: and (*c*) parasites with both nuclei and cytoplasm slightly or markedly distorted.

5. Red corpuscles to which distorted parasites are attached usually appear to be flattened out with slightly irregular outline, but parasites may be more or less distorted without any apparent change in the infected corpuscle.

6. Parasites with well stained chromatin and faded cytoplasm, or parasites with well stained cytoplasm and faded chromatin, may be found in specimens containing well stained parasites. It is usually possible to restain these specimens bringing out the faded cytoplasm belonging to the well stained chromatin or the lost chromatin belonging to the well stained cytoplasm.

EXPLANATION OF PLATES.

Plate 27.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,680. Tertian parasite (Fig. 35).

Figs. 1 to 8. Parasites secured 15 minutes after a paroxysm.

Figs. 9 to 14. Parasites secured 1 hour after a paroxysm.

Figs. 15 to 35. Parasites secured 4 hours after a paroxysm.

Fig. 1. Parasite attached to the surface of a corpuscle with a nucleus extending beyond the periphery of the corpuscle.

Fig. 2. Two parasites attached to one corpuscular mound, the nucleus of one parasite extending beyond the periphery of the corpuscle. It requires the entire cytoplasm of each parasite to encircle the mound, so that the cytoplasm of one parasite being superimposed over that of the other parasite gives the appearance of a single parasite with two masses of chromatin.

Figs. 3 to 5. Parasites attached to peripheral corpuscular mounds; the nuclei of these parasites extend beyond the periphery of the corpuscles to which they are attached.

Fig. 6. Parasites resting on the periphery of the corpuscles to which they are attached. Cytoplasm as well as nuclei may be seen extending beyond the periphery of the corpuscle.

Fig. 7. Two parasites attached to a peripheral corpuscular mound and two parasites attached to a surface mound. The nuclei of the parasites attached to the peripheral mound may be seen extending beyond the periphery of the corpuscle.

Fig. 8. Parasites attached to surface mounds. At the left two parasites encircle one mound; their cytoplasm being superimposed, they give the appearance of a single parasite with two masses of chromatin.

Fig. 9. Parasites attached to decolorized peripheral mounds. Portions of these parasites extend beyond the periphery of the corpuscle.

Fig. 10. Two parasites attached to one corpuscular mound; the cytoplasm of the upper parasite extends beyond the periphery of the corpuscle.

Fig. 11. Two parasites attached to a peripheral corpuscular mound, and two parasites of a slightly larger growth, attached in a similar manner to a surface corpuscular mound.

Figs. 12 and 13. Two parasites attached to one corpuscular mound. These parasites may be seen to be individuals.

Fig. 14. Four parasites attached to one corpuscular mound. A careful scrutiny will enable one to trace each parasite.

Figs. 15 to 20. Parasites attached to peripheral corpuscular mounds. The nuclei of the parasites in Figs. 18 and 19 may be seen extending beyond the periphery of the corpuscles.

Fig. 21. The parasite rests on the periphery of the corpuscle, and the pseudopodium may be seen encircling the peripheral corpuscular mound.

Fig. 22. Two parasites encircling one peripheral corpuscular mound.

Fig. 23. The parasite rests on the periphery of the corpuscle and encircles a corpuscular mound in front of it.

Figs. 24 to 33. Parasites resting on the periphery of the corpuscles with pseudopodia encircling the surface corpuscular mounds.

Fig. 34. Two parasites encircling one decolorized corpuscular surface mound.

Fig. 35. Three tertian parasites attached to a red corpuscle. One parasite surrounds with its pseudopodium the other parasite.

Plate 28.

TERTIAN PARASITES, EXTRACELLULAR EVIDENCE.

Magnification, × 1,680.

Figs. 36 to 41. Young parasites resting on the periphery of the corpuscles to which they are attached.

Fig. 42. A parasite attached to a peripheral corpuscular mound.

Fig. 43. A parasite resting on the periphery of a corpuscle with pseudopodia overlying the corpuscle.

Fig. 44. A parasite attached to a corpuscle. The nucleus and part of the cytoplasm of this parasite may be seen extending beyond the periphery of the corpuscle.

FIG. 45. A parasite resting on the periphery of the corpuscle to which it is attached.

FIGS. 46 to 49. Parasites partly pulled off the corpuscles to which they are attached. Technique is responsible for the dislocation.

FIG. 50. A young parasite attached to a corpuscle. The nuclei of the parasite are seen extending beyond the periphery of the corpuscle.

FIGS. 51 and 52. Two parasites partly removed from the corpuscles to which they are attached. The parasite in Fig. 51 is attached to a peripheral corpuscular mound, the parasite in Fig. 52 to a surface mound.

FIG. 53. A parasite attached to a red corpuscle with nuclei and pseudopodia (at o) extending beyond the periphery of the corpuscle.

FIG. 54. A pigmented parasite which has probably just attached itself to a fresh red corpuscle, the hemoglobin of which appears to be intact.

FIG. 55. A young parasite, unfolded and partly removed from the infected corpuscle by technique.

FIG. 56. A young parasite attached to a peripheral corpuscular mound with its pseudopodium extending beyond the periphery of the corpuscle.

FIG. 57. A pigmented parasite which is probably just attaching itself to a fresh red corpuscle, the hemoglobin of which appears to be intact.

FIG. 58. A young parasite with two pigment granules partly removed from a red corpuscle by technique. I believe that this parasite has recently attached itself as the hemoglobin of the corpuscle appears to be intact, while the parasite is pigmented.

FIGS. 59 to 61. Parasites resting on the periphery of the corpuscles to which they are attached. The parasites in Figs. 59 and 61 are pigmented and older than that in Fig. 60. The latter is unpigmented and flattened out by technique.

FIG. 62. A pigmented parasite partly pulled off the infected corpuscle by technique. This corpuscle shows no loss of hemoglobin though the parasite is heavily pigmented.

FIGS. 63 to 65. Adult pigmented parasites with portions of their cytoplasm extending beyond the periphery of the corpuscles to which they are attached.

FIG. 66. A parasite resting on the periphery of the corpuscle with its pseudopodium encircling a surface mound.

FIG. 67. An adult parasite attached to a corpuscle with the nucleus extending beyond the periphery of the corpuscle.

PLATE 29.

TERTIAN, ÆSTIVO-AUTUMNAL, AND QUARTAN PARASITES.

Magnification, × 1,680.

FIG. 68. A red corpuscle with Schüffner's granules and no parasite, from a case of tertian infection.

FIG. 69. A red corpuscle with Schüffner's granules and no parasite at o next to a red corpuscle with blue stippling, from a tertian infection.

Fig. 70. A red corpuscle with Schüffner's granules and no parasite, from a tertian infection.

Fig. 71. A red corpuscle with Schüffner's granules and no parasite next to a red corpuscle with Schüffner's granules and a tertian parasite.

Fig. 72. A free, distorted ring-form parasite from a tertian infection. The nucleus may be seen at x and a grain of pigment at o.

Fig. 73. A red corpuscle with Schüffner's granules and a tertian parasite next to a granular corpuscular skeleton or dehemoglobinized remnant of a red corpuscle. These examples are shown side by side so that the differences in granulations and staining reaction may readily be noted.

Fig. 74. A tertian parasite partly pulled off a red corpuscle with Schüffner's granules.

Fig. 75. A free, distorted ring-form parasite from a tertian infection. The nucleus may be seen at x and a grain of pigment at o.

Fig. 76. An adult tertian parasite partly pulled off a red corpuscle with Schüffner's granules.

Fig. 77. A crescent attached to a red corpuscle showing Schüffner's granules.

Fig. 78. A crescent attached to a red corpuscle showing Maurer's rings. The infected corpuscle is not entirely decolorized.

Fig. 79. An æstivo-autumnal ring-form parasite attached to a red corpuscle showing Maurer's rings and dots. The magnification of this figure is 2,120.

Fig. 80. Two young quartan parasites attached to a red corpuscle showing Maurer's rings and dots. The parasites are slightly distorted (in one direction) by technique.

Fig. 81. A quartan microgametocyte attached to a red corpuscle showing Schüffner's granules.

Fig. 82. A tertian segmenting body attached to an apparently healthy corpuscle. The chromatin is much distorted by technique without distortion of the infected corpuscle. The light dots are pigment granules that have been partially dissolved by xylol. This parasite must have received its pigment from another red corpuscle or corpuscles.

Fig. 83. A set of nuclei and pigment granules removed from a tertian parasite by technique. This example serves to illustrate the enormous amount of chromatin contained in the nuclei of young parasites. The long strands illustrated are drawn out from the chromatin of the nuclei.

Fig. 84. Two pigmented tertian parasites attached to a fairly healthy-appearing corpuscle. The nucleus of the parasite at the left may be seen to be distorted at x. The nucleus of the parasite at the right may be seen projecting beyond the periphery of the corpuscle at o.

Figs. 85 to 87. Young æstivo-autumnal parasites attached to red corpuscles with the chromatin of their nuclei drawn out in long thread-like processes beyond the periphery of the corpuscles to which the parasites are attached. These figures serve to illustrate not only the large amount of chromatin in the nuclei

of the young parasites but prove that these parasites are attached to the external surface of the corpuscles. In Fig. 86 at o two tiny parasites surround the corpuscular mound.

FIGS. 88 to 90. Chromatin dots (nuclei) of parasites whose cytoplasm has faded. Well stained crescents may be seen near the nuclei. A twin specimen, which had also faded, was successfully restained.

FIG. 91. A young æstivo-autumnal parasite, well stained, from the same specimen as that containing Figs. 88, 89, and 90.

PLATE 30.

ÆSTIVO-AUTUMNAL PARASITES PARASITES PARTIALLY FADED AND PARASITES DISTORTED BY TECHNIQUE.

Magnification, × 1,680.

FIGS. 92 to 97. Parasites with faded nuclei on corpuscles to which are attached parasites with well stained nuclei. The parasites with faded nuclei may be seen at o. In Fig. 96 at x a free parasite with stained nucleus may be seen.

FIG. 98. A distorted parasite at x on a corpuscle next to a corpuscle to which is attached another distorted parasite whose nucleus has faded until only a small particle of chromatin is to be seen at o.

FIG. 99. A well stained distorted parasite attached to a corpuscle next to a corpuscle to which is attached a parasite whose cytoplasm is fading.

FIG. 100. A parasite with faded nucleus at o attached to a corpuscle on which is a parasite with well stained nucleus and cytoplasm.

FIG. 101. Two parasites with distorted chromatin and fading cytoplasm.

FIG. 102. Two distorted parasites, the nucleus of one being faded, at o, and that of the other fading, attached to a corpuscle, next to a corpuscle to which are attached two well stained distorted parasites at x. Note that these four parasites are all distorted in one direction.

FIG. 103. Two parasites with well stained cytoplasm and faded chromatin.

FIG. 104. A distorted parasite with well stained cytoplasm and faded nucleus.

FIGS. 105 to 109. Chromatin masses of parasites whose cytoplasm has faded. These chromatin masses all show distortion by technique. In Fig. 105 at o may be seen two young parasites attached to one corpuscular mound. The nuclei of these parasites are fused so as to give the appearance of two rings with one mass of chromatin.

FIG. 110. Two young, unfaded parasites attached to a peripheral mound.

FIG. 111. Two parasites with faded cytoplasm attached to a peripheral mound. Compare with Fig. 110.

FIG. 112. A young parasite with faded cytoplasm. The nucleus is distorted and shaped to the peripheral mound to which the parasite is attached.

FIG. 113. Multiple infection of two surface mounds by young parasites.

FIG. 114. Chromatin dots (nuclei) of young parasites whose cytoplasm has faded. These nuclei are normal in shape, and from their position, in relation to each other (Fig. 113), multiple infection of corpuscular mounds is suggested.

FIG. 115. Young parasites distorted by technique.

FIG. 116. Distorted masses of chromatin. The cytoplasm of these parasites has faded.

FIG. 117. Parasites distorted by technique. At o two parasites are attached to one corpuscular mound, the nuclei being fused so as to give the appearance of two rings with a single mass of chromatin.

PLATE 31.

ÆSTIVO-AUTUMNAL PARASITES. FREE PARASITES AND PARASITES DISTORTED BY TECHNIQUE.

Magnification, × 1,680.

These parasites were secured 15 minutes after a paroxysm.

FIGS. 118 to 122. Tiny free parasites. At o in Fig. 119 two parasites may be seen surrounding one corpuscular mound. These parasites are from the same film as the normal and distorted parasites in Figs. 1 to 8.

FIGS. 123 and 124. Tiny free parasites at o, and parasites distorted and attached to corpuscles. In Fig. 124 one of the free parasites may be seen to have a pseudopodium.

FIG. 125. Distorted parasites and a multiple infection of a corpuscular mound at o.

FIGS. 126 to 129. Distorted parasites. The nuclei show more distortion than the cytoplasm. In Fig. 127 fusion of the chromatin of two parasites may be seen at o. In Fig. 128 at x, two parasites with distorted nuclei may be seen occupying one corpuscular mound.

FIG. 130. Multiple infection of corpuscular mounds. The parasites are distorted in one general direction.

FIGS. 131 to 142. Various types of distorted parasites. Figs. 131 and 140 at x show two ring-form parasites occupying one corpuscular mound. The nuclei of these parasites are distorted.

FIG. 143. Multiple infection of a red corpuscle. At o four young parasites may be seen attached to one corpuscular mound.

FIGS. 144 and 145. Distorted parasites.

FIG. 146. A free distorted parasite.

PLATE 32.

ÆSTIVO-AUTUMNAL PARASITES. PARASITES DISTORTED BY TECHNIQUE.

Magnification, × 1,680.

These parasites were secured 4 hours after a paroxysm, and from the same case as the normal parasites seen in Figs. 15 to 34.

FIG. 147. Three young parasites attached to one corpuscle and similarly distorted.

FIG. 148. Distorted parasites. At o a peripheral corpuscular mound may be seen. These parasites are distorted in one general direction.

FIG. 149. Two young parasites attached to one corpuscle and similarly distorted.

FIGS. 150 to 152. Distorted parasites. Each figure shows the parasites distorted in one general direction. At x in Fig. 151 the chromatin is intact and the cytoplasm distorted.

FIGS. 153 and 154. Parasites similarly distorted. At x in Fig. 154 the cytoplasm is normal in appearance, the chromatin distorted.

FIG. 155. Distorted parasites. The extraneous thread at o extends across the field in the same general direction as the distorted parasites.

FIG. 156. Three parasites attached to one corpuscle. All three parasites are distorted in one general direction.

FIG. 157. Three parasites distorted in one general direction.

FIG. 158. Two parasites on one corpuscle. These parasites are distorted in the same general direction as the single parasite attached to the corpuscle at the left.

FIGS. 159 to 162. Parasites distorted in one general direction. In Fig. 161 two parasites are attached to one of the corpuscles. The pseudopodia of one of these parasites may be seen extending beyond the periphery of the infected corpuscle at o.

FIG. 163. A free parasite, normal in appearance, of the same age and from the same film as the distorted parasites.

FIG. 164. A parasite with distorted cytoplasm. A portion of the cytoplasm may be seen extending beyond the periphery of the infected corpuscle at o.

FIG. 165. Parasites with distorted cytoplasm. The parasites are distorted in one direction.

FIG. 166. Parasites with distorted chromatin and cytoplasm. These parasites are distorted in one direction.

FIG. 167. Three parasites similarly distorted.

PLATE 33.

ÆSTIVO-AUTUMNAL, QUARTAN, AND TERTIAN PARASITES DISTORTED BY TECHNIQUE.

Magnification, × 1,680.

Figs. 168 to 182. Æstivo-autumnal parasites secured 1 hour after a paroxysm.

Figs. 183 to 192. Quartan parasites in the same stage of development as the parasites in Fig. 80.

Figs. 193 to 202. Tertian parasites.

FIG. 168. The chromatin of this parasite is distorted to conform to the contour of the decolorized peripheral corpuscular mound.

FIG. 169. At o two young parasites are attached to one corpuscular mound, and the chromatin is distorted and fused so as to give the appearance of two rings with a single mass of chromatin.

FIG. 170. Multiple infection of a corpuscular mound. The cytoplasm of these parasites is more distorted than the chromatin.

FIGS. 171 and 172. Two parasites attached to each corpuscle. The parasites are distorted in one direction.

FIGS. 173 to 176. Parasites with cytoplasm and chromatin distorted.

FIGS. 177 to 180. Two and three (Fig. 180) parasites attached to one corpuscle and distorted in one direction.

FIG. 181. A distorted free parasite.

FIG. 182. A free parasite fairly normal in appearance.

FIGS. 183 to 187. Distorted parasites. The infected red corpuscles appear normal in contour.

FIG. 188. Two parasites attached to one corpuscular mound and distorted, both in one direction.

FIG. 189. Two parasites which were probably attached to one corpuscular mound. These parasites are distorted in one direction.

FIGS. 190 to 192. Distorted parasites.

FIGS. 193 to 196. Parasites with distorted cytoplasm.

FIG. 197. Two parasites attached to one corpuscle. The cytoplasm of these parasites is distorted in one direction.

FIG. 198. Two parasites attached to one corpuscular mound. The cytoplasm is distorted and the nuclei are fused.

FIGS. 199 to 202. Parasites with distorted chromatin and cytoplasm.

PLATE 34.

TERTIAN PARASITES. PARASITES DISTORTED BY TECHNIQUE.

Magnification, × 1,680.

FIG. 203. A distorted and a normal parasite attached to one corpuscle. The nucleus of the normal parasite may be seen to project beyond the periphery of the infected corpuscle at o. A careful scrutiny of the picture will show that this parasite is on the under surface of the infected corpuscle.

FIG. 204. A distorted parasite. A peripheral corpuscular mound may be seen at o. The distorted parasite was probably pulled away from this mound by technique, as the mound is seen to be partly decolorized.

FIG. 205. A normal ring-form parasite from the same specimen as that containing many of the distorted forms.

FIG. 206. Multiple infection of a corpuscular mound. The cytoplasm and chromatin are distorted.

FIG. 207. Multiple infection of a corpuscular mound by two parasites. The cytoplasm is distorted.

FIGS. 208 to 223. Various types of distorted parasites.

FIGS. 224 and 225. Two types of distorted free parasites.

FIG. 226. Parasites with both chromatin and cytoplasm distorted. At x two distorted parasites may be seen occupying one corpuscular mound.

Figs. 227 to 230. Two parasites attached to each corpuscle. These parasites are distorted in one direction.

Fig. 231. Three or more parasites attached to one corpuscle. These parasites are attached and distorted in such a manner that it is impossible to be sure how many parasites are present.

Figs. 232 to 236. Adult parasites distorted. These parasites are flattened out and drawn across the infected corpuscle. They correspond to the so called "*bandform*" parasites described by many observers as present in quartan infections.

Plate 35.

TERTIAN AND ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,680.

Fig. 237. (Corresponds to Fig. 18.) An æstivo-autumnal parasite attached to a peripheral corpuscular mound. The nucleus extends beyond the periphery of the infected corpuscle.

Fig. 238. (Corresponds to Fig. 11.) Æstivo-autumnal parasites. Two are attached to a peripheral corpuscular mound, and two to a surface corpuscular mound.

Fig. 239. (Corresponds to Fig. 32.) Æstivo-autumnal parasites. One rests on the periphery of the corpuscle with its pseudopodium overlying the red corpuscle in the form of a loop; the other is attached to a surface mound.

Fig. 240. (Corresponds to Fig. 38.) A tertian parasite resting on the periphery of a corpuscle with pseudopodia overlying the surface of the corpuscle.

Fig. 241. (Corresponds to Fig. 39.) A tertian parasite resting on the periphery of a corpuscle with pseudopodia overlying the surface of the corpuscle.

Fig. 242. (Corresponds to Fig. 42.) A tertian parasite resting on the periphery of a corpuscle, attached to a peripheral corpuscular mound.

Fig. 243. (Corresponds to Fig. 43.) A tertian parasite resting on the periphery of a corpuscle with pseudopodia overlying the surface of the corpuscle.

Fig. 244. (Corresponds to Fig. 45.) A tertian parasite resting on the periphery of the corpuscle with its pseudopodium overlying the surface of the infected corpuscle.

Fig. 245. (Corresponds to Fig. 61.) A pigmented tertian parasite resting on the periphery of a red corpuscle with pseudopodia overlying the infected corpuscle.

Fig. 246. (Corresponds to Fig. 62.) A pigmented tertian parasite partly pulled off a red corpuscle.

Fig. 247. (Corresponds to Fig. 57.) A pigmented tertian parasite attaching itself to a fresh red corpuscle.

Fig. 248. (Corresponds to Fig. 54.) A pigmented tertian parasite attaching itself to a fresh red corpuscle.

Fig. 249. (Corresponds to Fig. 52.) A pigmented tertian parasite partly pulled off a red corpuscle showing degenerative changes caused by the parasite.

FIG. 250. (Corresponds to Fig. 63.) A pigmented tertian parasite with its pseudopodium extending beyond the periphery of the infected corpuscle.

FIG. 251. (Corresponds to Fig. 138.) Æstivo-autumnal parasites with chromatin distorted by technique.

FIG. 252. (Corresponds to Fig. 123.) Æstivo-autumnal parasites. A free parasite may be seen at o. Distorted parasites are attached to the corpuscle.

FIG. 253. (Corresponds to Fig. 51.) A pigmented tertian parasite partly pulled off the infected corpuscle. The peripheral mound to which the parasite is attached may be seen at o. The corpuscle shows slight degenerative changes owing to the presence of the parasite.

FIG. 254. (Corresponds to Fig. 48.) A pigmented parasite partly pulled off the infected red corpuscle.

FIG. 255. (Corresponds to Fig. 130.) Distorted æstivo-autumnal parasites.

FIG. 256. (Corresponds to Fig. 139.) Distorted æstivo-autumnal parasites.

FIG. 257. (Corresponds to Fig. 144.) Distorted æstivo-autumnal parasites.

FIG. 258. (Corresponds to Fig. 132.) Distorted æstivo-autumnal parasites.

FIG. 259. (Corresponds to Fig. 137.) Distorted æstivo-autumnal parasites.

FIG. 260. (Corresponds to Fig. 145.) Distorted æstivo-autumnal parasites.

FIG. 261. (Corresponds to Fig. 141.) Distorted æstivo-autumnal parasites.

FIG. 262. (Corresponds to Fig. 142.) Distorted æstivo-autumnal parasties.

FIG. 263. (Corresponds to Fig. 133.) Distorted æstivo-autumnal parasites.

FIG. 264. (Corresponds to Fig. 87.) An æstivo-autumnal parasite with the chromatin of the nucleus drawn out in a long thread-like process far beyond the periphery of the infected corpuscle. This illustrates the large amount of chromatin in the nuclei of young parasites.

FIG. 265. (Corresponds to Fig. 75.) A free distorted tertian ring-form parasite.

FIG. 266. (Corresponds to Fig. 107.) Distorted chromatin of æstivo-autumnal parasites whose cytoplasm has faded.

FIG. 267. (Corresponds to Fig. 116.) Distorted chromatin of æstivo-autumnal parasites whose cytoplasm has faded.

FIG. 268. (Corresponds to Fig. 103.) Cytoplasm of æstivo-autumnal parasites whose chromatin has faded.

FIG. 269. (Corresponds to Fig. 114.) Chromatin dots (nuclei) of æstivo-autumnal parasites whose cytoplasm has faded. These nuclei are normal in appearance.

315

THE FORMATION OF METHEMOGLOBIN BY STREPTOCOCCUS VIRIDANS.

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Certain strains of streptococci, when grown on blood agar plates, produce a green color about the colonies, a characteristic which serves to differentiate them from other strains which produce a clear hemolytic zone. It has long been recognized that the change produced in the blood by these streptococci is a transformation of oxyhemoglobin to methemoglobin, and it is this metabolic function which serves to identify them as belonging to the type now generally called *Streptococcus viridans*.

A similar transformation of oxyhemoglobin to methemoglobin is caused by the pneumococcus. Cole[1] has studied the nature of this reaction and has found that it occurs only when the pneumococci are living and, furthermore, only in the presence of certain nutritive materials, although this material need be present only in very minute quantities. He concludes that the process is due to the alteration of the oxidative processes by the pneumococci in the vicinity of the red blood cells and not to the production of any injurious substance capable of isolation; and therefore that the pathological effects of bacteria on other tissue cells may be due to disturbances in oxidation in the immediate neighborhood of the bacteria and not necessarily to the action of a definite poison.

The part which *Streptococcus viridans* plays in human pathology is becoming increasingly evident and for that reason it has seemed of value to investigate its method of action. The nature of the reaction during which methemoglobin is formed is readily available for study, and it has been the purpose of this work to determine whether this function of *Streptococcus viridans* is the same as that possessed by the pneumococcus.

[1] Cole, R., *J. Exp. Med.*, 1914, xx, 363.

Methods.

Measured amounts of cultures of *Streptococcus viridans* were added to 1 cc. of a 5 per cent normal saline suspension of washed sheep red blood corpuscles in small test-tubes under many different environmental conditions. These mixtures with suitable controls were incubated in a water bath at 37°C. for varying periods of time as the requirements of the experiments demanded. Methemoglobin formation was considered to have taken place when the red color of oxyhemoglobin was changed to the brown color of methemoglobin, and the results have been recorded throughout as follows: ++ indicates complete transformation of oxyhemoglobin to methemoglobin; + indicates moderate methemoglobin formation; +− indicates slight methemoglobin formation; − indicates no demonstrable change.

Preliminary experiments showed that the most satisfactory culture medium for use was plain ascitic bouillon (peptone 2 per cent, bouillon 4 parts, ascitic fluid 1 part, reaction 0.3+ to phenolphthalein) and this has been used throughout. Such a medium in itself has no effect on sheep red blood corpuscles; *Streptococcus viridans* grows abundantly and rapidly in it; and an acidity sufficient in itself to transform oxyhemoglobin into methemoglobin is never produced, such as occurs when a culture medium containing dextrose is used.

Many different strains of *Streptococcus viridans* have been tested, isolated from various sources, such as acute tonsillitis, bronchopneumonia, malignant endocarditis, alveolar abscesses, etc. No significant difference in the action of different strains has occurred.

EXPERIMENTAL.

To determine whether living streptococci were necessary to cause methemoglobin formation, paralle. tests with living cultures, cultures killed by exposure to 58°C. for 30 minutes, and with culture medium from which the streptococci had been removed by centrifugalization were made. The results are shown in Table I.

These experiments have been repeated many times and have constantly shown that methemoglobin is formed only in the presence

TABLE I.

Tube No.	Material used.	Methemoglobin formation, 2 hrs. at 37°C.
1	1 cc. Culture E3 + 1 cc. suspension of sheep corpuscles.	++ in 10 min.
2	1 cc. Culture E3 (heated at 58°C. for 30 min.) + 1 cc. suspension of sheep corpuscles.	–
3	1 cc. Culture E3 (streptococci removed) + 1 cc. suspension of sheep corpuscles.	–
4	1 cc. sterile culture media + 1 cc. suspension of sheep corpuscles.	–

Culture E3 is an 18 hr. ascitic bouillon culture of *Streptococcus viridans* E3 from a case of malignant endocarditis.

of living streptococci and not by killed organisms or by any substance produced by the growth of the bacteria in the culture medium and separable from them.

To determine the optimum period of growth for the production of methemoglobin and to discover whether this function of *Streptococcus viridans* persisted as long as the organisms were viable, or was associated only with multiplication of the bacteria, the following experiment was done. Measured amounts of culture were added to washed sheep corpuscles at frequent intervals up to 60 hours after inoculation of the culture medium, and results recorded in the usual way as shown in Table II.

The formation of methemoglobin occurred after the culture had been incubated for 6 hours and continued at its height up to 18 hours. After this time the intensity of the reaction gradually diminished and finally disappeared after 60 hours' incubation. Subcultures made at this time showed the culture to be still viable. Numerous similar experiments have yielded like results with some slight variation in the optimum period for the most active formation of methemoglobin. These variations have apparently depended upon the rapidity of growth of a given strain. From these experiments it seems probable that the reaction is associated with multiplication of the streptococci, being most intense during the period of greatest growth and

TABLE II.

Tube No.	Culture T1.	Salt solution.	Sheep corpuscles.	Methemoglobin formation, 1 hr. at 37°C., after incubation of Culture T1 from 3 to 60 hrs.								
				Hours.								
				3	6	9	14	18	23	30	48	60
	cc.	*cc.*	*cc.*									
1	1.0	—	1.0	—	++	++	++	++	++	+	+	—
2	0.9	0.1	1.0	—	++	++	++	++	++	+	+	—
3	0.8	0.2	1.0	—	++	++	++	++	+	+	+−	—
4	0.7	0.3	1.0	—	++	++	++	++	+	+	+−	—
5	0.6	0.4	1.0	—	+	++	++	++	+	+−	+−	—
6	0.5	0.5	1.0	—	+	+	+	+	+−	+−	+−	—
7	0.4	0.6	1.0	—	+−	+	+	+	+−	+−	+−	—
8	0.3	0.7	1.0	—	—	+−	+−	+−	+−	—	—	—
9	0.2	0.8	1.0	—	—	+−	+−	+−	—	—	—	—
10	0.1	0.9	1.0	—	—	—	—	—	—	—	—	—
11	—	1.0	1.0	—	—	—	—	—	—	—	—	—
12	1. cc. of sterile culture media.	—	1.0	—	—	—	—	—	—	—	—	—

gradually disappearing as further multiplication ceases, though the culture still remains viable.

In testing various strains of *Streptococcus viridans* in the preceding experiment it soon became evident that there was considerable variation in the ability of various strains to form methemoglobin. Much larger amounts of culture were required with some strains than with others to cause complete transformation of oxyhemoglobin to methemoglobin when tested against a constant amount of sheep corpuscles. It seemed possible that this variability might bear some relation to the virulence of the strains or to the sources from which they were isolated. Accordingly titration of a considerable number of strains was done under uniform conditions. Diminishing amounts of 12 hour ascitic bouillon cultures were added to 1 cc. of washed sheep corpuscles, and incubated for 1 hour at 37°C. The smallest amount of culture which caused complete transformation of oxyhemoglobin to methemoglobin was taken as a measure of the activity of a given strain and may be spoken of as the methemoglobin titer of that strain. A tabulation of the results obtained, with the source and virulence of the strains for comparison, is shown in Table III.

TABLE III.

Culture.	Methemoglobin titer.	Source.	Virulence for rabbits.*	
			Amount of culture.	Effect.
	cc.		cc.	
AA3b3....	1.0	Alveolar abscess.		
T12d......	1.0	Acute tonsillitis.	12	No effect.
			40	Septicemia; death in 18 hrs.
SF1.......	0.9	Throat, scarlet fever.	5	No effect.
R1........	0.8	" rheumatic fever.	40	Arthritis.
T5........	0.7	Acute tonsillitis.	10	Septicemia; death in 20 hrs.
T14.......	0.7	" "	4	No effect.
			12	Septicemia; death in 18 hrs.
AA3a......	0.6	Alveolar abscess.		
AA3b1....	0.6	" "		
T1........	0.6	Acute tonsillitis.	5	Acute pericarditis, acute myocarditis, arthritis.
AA3b4....	0.5	Alveolar abscess.		
D1a.	0.5	Throat, diphtheria.		
AA1k.....	0.5	Alveolar abscess.	5	Aortic endocarditis, myocarditis, arthritis; death in 18 days.
P8........	0.4	Pneumonia.	2	No effect.
P10.......	0.4	"	5	Arthritis.
AA1.......	0.4	Alveolar abscess.	2	Septicemia; death in 24 hrs.
AA1j......	0.4	" "		
T3........	0.3	Acute tonsillitis.	60	Mitral endocarditis, appendicitis, arthritis; death in 9 days.
T4........	0.3	" "	15	Septicemia; death in 24 hrs.
T14b	0.3	" "		
E12b......	0.3	Blood, endocarditis.		
T11.......	0.2	Acute tonsillitis.	5	Acute pericarditis, acute arthritis; death in 24 hrs.
T11a......	0.2	Abscess, pyemia.		
D1c.......	0.2	Throat, diphtheria.		
P3........	0.2	Pneumonia.	4	Septicemia; death in 18 hrs.
AA2.......	0.2	Alveolar abscess.	8	No effect.
E3........	0.2	Blood, endocarditis.	50	Arthritis.
E12v2.....	0.2	Mitral valve, endocarditis.		
T12b......	0.1	Acute tonsillitis.		
T12c......	0.1	" "		

* Rabbit inoculations were given intravenously. Measured amounts of 24 hr. dextrose bouillon cultures were centrifuged and the bacterial sediment was suspended in salt solution before inoculation.

Study of the results shows no relation between the source and virulence of the different strains of *Streptococcus viridans* and their methemoglobin titers. Similar results have been found for hemolytic streptococci by Lyall.[2] On the other hand, the titer of our strains seemed to vary largely with the abundance and rapidity of growth of a given strain, thus lending further support to the theory that the formation of methemoglobin is a function associated with multiplication of the streptococci.

If the formation of methemoglobin is the result of the metabolic activities occurring during the growth and multiplication of the streptococci, the reaction should take place only under those conditions which are suitable for multiplication; that is, in the presence of nutrient material. The following experiment has shown this to be true. An 18 hour ascitic broth culture of streptococci was centrifuged, the supernatant fluid removed, and the bacterial sediment thoroughly washed and finally suspended in salt solution, the suspension being prepared so that the streptococci were in twice as great concentration as in the original culture. This suspension was tested against sheep corpuscles alone and with the addition of ascitic bouillon, plain bouillon, ascitic fluid, meat infusion, peptone, and dextrose as shown in Table IV.

It was evident from these experiments that some nutrient substance is necessary for the formation of methemoglobin. Repetition of these experiments with increasing dilutions of the various substances showed that relatively large amounts of ascitic fluid, meat infusion, and peptone were required for the reaction, but that methemoglobin was formed rapidly in the presence of dextrose in great dilution, even in as small amounts as 0.5 cc. of a 1: 1,000 dilution of a 5 per cent dextrose solution. Since the presence of such minute traces of dextrose is sufficient to bring about the reaction, it seems possible that traces of sugar may have been present in the peptone solution and have been responsible for the reaction in that case. Certainly this may have been so in the experiments with ascitic fluid and meat infusion.

Various kinds of sugars and other substances were next tested to determine whether streptococci would produce methemoglobin in their

[2] Lyall, H. W., *J. Med. Research*, 1914, xxx, 515.

TABLE IV.

Tube No.	Material used.	Methemoglobin formation, 2 hrs. at 37°C.
1	0.5 cc. bacterial suspension T1+1 cc. sheep corpuscles +0.5 cc. salt solution.	–
2	0.5 " " " T1+1 " " " +0.5 " ascitic bouillon.	++
3	0.5 " " " T1+1 " " " +0.5 " plain "	++
4	0.5 " " " T1+1 " " " +0.5 " ascitic fluid.	++
5	0.5 " " " T1+1 " " " +0.5 " meat infusion.	++
6	0.5 " " " T1+1 " " " +0.5 " peptone solution.	+
7	0.5 " " " T1+1 " " " +0.5 " dextrose "	++
8	0.5 " salt solution +1 " " " +0.5 " ascitic bouillon.	–
9	0.5 " " " +1 " " " +0.5 " plain "	–
10	0.5 " " " +1 " " " +0.5 " ascitic fluid.	–
11	0.5 " " " +1 " " " +0.5 " meat infusion.	–
12	0.5 " " " +1 " " " +0.5 " peptone solution.	–
13	0.5 " " " +1 " " " +0.5 " dextrose "	–
14	0.5 " Culture T1 +1 " " " +0.5 " salt "	++

The peptone solution consisted of 5 per cent solution of Witte's peptone in distilled water, autoclaved for 15 minutes at 15 pounds' pressure.

The dextrose solution consisted of 5 per cent solution of chemically pure dextrose in distilled water, autoclaved for 15 minutes at 15 pounds' pressure.

presence as well as in the presence of dextrose. The results are given in Table V. Control tubes without the addition of streptococci showed that none of the test substances cause methemoglobin formation.

Further experiments with increasing dilutions of the various test substances to determine the minimum amount of each substance in the presence of which the reaction would occur showed considerable variation not only in the amount required but also in the rapidity of the change of oxyhemoglobin to methemoglobin. No definite relationship could be determined between these variable results and the molecular configuration of the substances used, although the reaction was usually more rapid and occurred in the presence of higher dilutions of dextrose, lactose, saccharose, maltose, and levulose than of the other substances. Furthermore, there seemed to be no relationship between the carbohydrate cleavage power of a strain

TABLE V.

Test substance.	Bacterial suspension.	Sheep corpuscles.	Methemoglobin formation.		
			T3.	P 10.	E3.
	cc.	*cc.*			
0.5 cc. 3 per cent inulin solution	0.5	1.0	+	++	++
0.5 " 5 " " lactose "	0.5	1.0	++	++	+−
0.5 " 5 " " mannite "	0.5	1.0	+	++	−
0.5 " 5 " " raffinose "	0.5	1.0	++	++	++
0.5 " 5 " " saccharose "	0.5	1.0	+	++	++
0.5 " 5 " " salicin "	0.5	1.0	+−	+	+−
0.5 " 5 " " galactose "	0.5	1.0	+	++	−
0.5 " 5 " " maltose "	0.5	1.0	++	++	++
0.5 " 5 " " dextrin "	0.5	1.0	++	++	++
0.5 " 5 " " levulose "	0.5	1.0	++	++	++
0.5 " 5 " " starch "	0.5	1.0	++	+	+
0.5 " 5 " " glycerine "	0.5	1.0	−	++	+
0.5 " 5 " " urea "	0.5	1.0	−	−	−
0.5 " 10 " " methyl alcohol	0.5	1.0	−	−	−
0.5 " 10 " " ethyl "	0.5	1.0	+	++	+

All the tubes were incubated at 37° C. for 2 hrs. T3, P10, and E3 are different strains of *Streptococcus viridans.*

of streptococcus and its ability to form methemoglobin in the presence of various carbohydrates. Of six strains which formed methemoglobin in the presence of inulin, three fermented the sugar, three did not; and of five strains causing methemoglobin formation in the presence of mannite, only one fermented that substance.

Since methemoglobin may be formed by streptococci in the presence of sugars which the organisms do not ferment, it seems improbable that the reaction depends upon intermediate or end-products formed by the action of the streptococci on the nutrient substance present. Certainly the reaction cannot be due to the formation of carbon dioxide, for carbon dioxide changes oxyhemoglobin to a cherry-red color and such a change is never caused by *Streptococcus viridans.* Neither can the reaction be due to the production of acid, for if experiments such as the above are carried out with the addition of a mixture of primary and secondary potassium phosphates so that neutrality of the mixture is maintained, not only does the formation of methemoglobin still take place, but in many instances it is acceler-

ated. Cole[1] has shown that many substances which are supposed to represent the intermediate and end-products of sugar metabolism are incapable of producing methemoglobin except in much more concentrated solutions than could possibly occur in the above experiments. It is conceivable that some of these substances might be sufficiently active in a nascent state to bring about the reaction, but this is hardly probable.

To determine the effect of the absence of oxygen on the reaction the following experiment was done.

A stream of hydrogen was passed through a suspension of red blood corpuscles for 10 minutes and the suspension then covered with paraffin oil. An ascitic bouillon culture of streptococci was treated in the same manner. A measured amount of the culture was then drawn into a pipette which had been passed through the oil to the bottom of the culture tube and was immediately added to the suspension of red blood cells, the pipette being passed through the oil to the bottom of the tube containing the corpuscle suspension. No air was allowed to enter the mixture. Mixtures so prepared showed no methemoglobin formation at the end of 2 hours' incubation, while in the untreated control tubes complete transformation of oxyhemoglobin to methemoglobin took place in 10 minutes. A stream of oxygen was now passed through the mixture previously treated with hydrogen, with the result that methemoglobin formation rapidly occurred. Two tubes were then prepared each containing a like mixture of the corpuscle suspension and an ascitic bouillon culture of streptococci. Through one a stream of oxygen was passed, through the other a stream of air. The tubes were incubated together. Under these conditions methemoglobin formation in the tube subjected to an excess of oxygen was considerably delayed. These experiments show that methemoglobin formation by *Streptococcus viridans* does not occur in the absence of oxygen and that it is retarded in the presence of an excess of oxygen.

It seemed possible that some further knowledge of the nature of the reaction during which methemoglobin is formed might be gained by studying the inhibitory action of various substances. Experiments were done in which diminishing amounts of toluol, formaldehyde, sodium salicylate, salicylic acid, potassium carbonate, potassium iodide, alcohol, and chloroform, and various sera were added to the culture-sheep-corpuscle mixtures. The results in general were not of sufficient significance to make it profitable to present them in detail. In brief it may be said that toluol (1:20), formaldehyde (1:2,000), 5 per cent sodium salicylate solution, and 20 per cent saturated salicylic acid solution had no appreciable effect on the reaction. Chloroform in the proportion of 1:20, ethyl alcohol in

the proportion of 1: 10, 100 per cent potassium iodide solution in the proportion of 1: 5, and 25 per cent potassium carbonate solution in the proportion of 1: 5 caused complete inhibition of the reaction. Inhibition of methemoglobin formation in the presence of these substances may be attributed to a reduction of the metabolic activities of the organisms to a minimum.

The results with various sera were interesting but threw little light on the mechanism of the reaction in question. Normal human sera as a rule caused definite retardation and partial inhibition of the reaction (Table VI).

TABLE VI.

	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Culture P3...	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1
Human serum.	0.5	0.4	0.3	0.2	0.1	0.5 (1: 10)	0.3 (1: 10)	0.1 (1: 10)	0.5	–
Salt solution..	0.4	0.5	0.6	0.7	0.8	0.4	0.6	0.8	0.5	0.9
Sheep corpuscles........	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Methemoglobin formation at 37°C.										
30 min.....	–	–	+–	+–	+–	+	+	+	–	+
1 hr........	+–	+–	+	+	+	++	++	++	–	++

In other instances the result was much less marked. Lyall[2] has called attention to the inhibiting effect of salvarsanized serum on the action of hemolytic streptococci. In our experiments salvarsanized serum was not more efficacious than normal human serum in its inhibitory action on methemoglobin formation. Normal sheep serum and guinea pig serum had a moderate inhibiting action, while normal rabbit serum had little effect.

Two experiments with sera from patients with streptococcus septicemia are of interest in this connection. A culture of *Streptococcus viridans* E3 obtained from the blood of a patient with malignant endocarditis was tested in the usual manner against sheep corpuscles with the addition of diminishing amounts of the patient's serum, a parallel series of tubes with normal human serum being run at the same time as a control (Table VII). Complete inhibition of methemoglobin formation took place in those tubes to which the patient's serum had been added.

TABLE VII.

Culture E3.	Salt solution.	Sheep corpuscles.	Serum.	Methemoglobin formation at 37°C.			
				Patient's serum.		Normal human serum.	
				30 min.	1 hr.	30 min.	1 hr.
cc.	*cc.*	*cc.*	*cc.*				
0.3	0.2	1.0	0.5	–	–	+–	++
0.3	0.3	1.0	0.4	–	–	+–	++
0.3	0.4	1.0	0.3	–	–	+	++
0.3	0.5	1.0	0.2	–	–	+	++
0.3	0.6	1.0	0.1	–	–	++	++
0.3	0.2	1.0	0.5 (1:10)	–	–	++	++
0.3	0.4	1.0	0.3 (1:10)	–	–	++	++
0.3	0.6	1.0	0.1 (1:10)	–	–	++	++
0.3	0.7	1.0	–	++	++	++	++
–	0.5	1.0	0.5	–	–	–	–

The second experiment in which similar results were obtained was with a hemolytic streptococcus isolated from the blood in a case of septicemia following tonsillectomy. Nearly complete inhibition of hemolysis occurred in the series of tubes to which the patient's serum had been added. This inhibition of the metabolic activities of the streptococci by serum may be concerned with the phenomena of antiblastic immunity as described by Dochez and Avery.[3]

SUMMARY AND CONCLUSIONS.

Cultures of *Streptococcus viridans* when brought into contact with red blood corpuscles have the power of transforming oxyhemoglobin into methemoglobin. The reaction occurs only in the presence of living streptococci when they are able to carry on their metabolic activities. The intensity of the reaction runs roughly parallel with the period of growth and multiplication of the bacteria and gradually diminishes and disappears as growth ceases. There is no apparent relation between the activity of a given strain of *Streptococcus viridans* in producing methemoglobin and its source or virulence. If the streptococci are suspended in salt solution they are unable to change

[3] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

oxyhemoglobin into methemoglobin unless some nutrient substance is present. Of the various nutrient substances tested dextrose is the most efficient in enabling the organisms to bring about the reaction. The reaction does not occur in the absence of oxygen, and is retarded by an excess of oxygen. Substances which tend to reduce the metabolic activities of the bacteria to a minimum exert an inhibitory action on methemoglobin formation. While not definitely proving it to be so, the results obtained in the above experiments strongly support the supposition that the reaction is not due to injurious substances produced by the bacteria or to products arising from the decomposition of the nutrient material present, but rather to the metabolic activities of the bacteria themselves when they are surrounded by environmental conditions which render growth and multiplication possible.

The exact chemical nature of the change of oxyhemoglobin to methemoglobin is not known, but it is probably an oxidation process or a combination of reduction and oxidation processes, as pointed out by Heubner.[4] As Cole[1] has shown, the action of aminophenol is of great interest in this connection, in that it acts like a catalytic agent in being able to transform much more hemoglobin into methemoglobin than would be possible if the reaction were a simple molecular one.

The metabolic activities of bacteria are largely in the nature of oxidation and reduction processes. The transformation of oxyhemoglobin into methemoglobin by streptococci of the *viridans* type, therefore, may be analogous to the action of such substances as aminophenol, and the reaction may be due to the active oxidation and reduction processes occurring in the neighborhood of the bacterial cells. The failure of the reaction to occur in the absence of oxygen and its retardation in the presence of an excess of oxygen, both with streptococci and with pneumococci (Cole[1]) would seem to support this theory. Such results, however, may be due to the abnormal conditions surrounding the bacteria with consequent inhibition of their metabolic activities. Cole[1] concluded as the result of his study of methemoglobin formation by pneumococci that since

[4] Heubner, W., *Arch. exp. Path. u. Pharm.*, 1913, lxxii, 239.

bacteria may injure red blood cells apparently by disturbances in oxidation in the immediate neighborhood of the organisms rather than by the production of a definite toxin, it is possible that bacteria may injure other tissue cells in a like manner and that the pathological effects produced by these bacteria may be explained on this basis. The experimental results recorded above have shown that the formation of methemoglobin by *Streptococcus viridans* in no way differs from its formation by pneumococci, and they lend support to the theory that bacteria may be injurious to tissues because of the disturbances in oxidation brought about by the metabolic activities of the organisms, especially those associated with growth and multiplication. It is believed that this theory may be particularly applicable to the pathological effects caused by *Streptococcus viridans* because the lesions produced by it, whether single or multiple, both in man and in experimental animals, are prone to be localized and associated with the actual presence of the streptococci in the lesions.

A FURTHER STUDY OF THE GASTRIC ULCERS FOLLOWING ADRENALECTOMY.

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PLATES 36 AND 37.

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In a previous study of adrenalectomized animals,[1] the frequent occurrence of acute ulceration of the gastric mucosa and the occasional occurrence of duodenal ulcer were noted. While these ulcers were not found in adrenalectomized animals subjected to continuous etherization, and were infrequent in animals subjected to the removal of only one gland, they occurred in about 90 per cent of those dying under the characteristic symptoms of adrenal insufficiency after the removal of both glands. The ulcers developed during the moribund period, were apparently peptic, forming at the site of the local hemorrhages in the gastric mucosa, and were true acute ulcers, usually penetrating to the muscularis mucosa, with a total loss of epithelium. While they occurred in the absence of pancreatic secretion and bile, they appeared to develop only in an acid medium.

In order to determine whether the acid medium was the important factor in the production of the acute ulcers, or whether their cause resided in other factors, such as the special nerve or vascular mechanism of the stomach, the following experiments were performed.

A loop of the first part of the jejunum, varying in length from 6 to 12 cm., was functionally resected under anesthesia and implanted in the posterior wall of the stomach in the region of the antrum pylori. The continuity of the intestine was maintained by an intestinal anastomosis.

[1] Mann, F. C., A Study of the Gastric Ulcers Following Removal of the Adrenals, *J. Exp. Med.*, 1916, xxiii, 203.

Dogs operated on in this manner quickly recovered from the operation and maintained excellent health for many months. Animals killed at various periods after operation demonstrated that the transplanted mucosa underwent definite changes, but ulcers did not develop except in one instance in which an ulcer was found around a retained silk suture.

In four of these animals, after a considerable length of time had elapsed, the right adrenal was removed, and a few months later the left gland was extirpated. All developed the typical symptoms of adrenal insufficiency and died at various times after the removal of the last gland.

In all these animals definite lesions of the gastric mucosa were found at autopsy. In three, there were ulcerations, while in one the mucosa was injected only and showed areas of erosion. In three the jejunal transplant appeared exactly similar to the control. In one there appeared to be a slight loss of jejunal mucosa in pin-point areas, which microscopically proved to be small ulcers.

It was hoped that these experiments would prove clearly whether or not free acidity was the primary cause in the formation of these ulcers. If acidity is the primary cause, the jejunal mucosa, to which an acid medium is foreign, should show the most marked changes. If the primary cause lies in the intrinsic mechanism of the gastric mucosa, the latter alone should be involved.

The results of the experiments show that both factors are of importance. In the three experiments in which the gastric mucosa alone was involved, the transplant having been left intact, either the ulcerations and erosions were due to a primary impairment of the gastric mucosa to which the jejunal mucosa was not subjected, or the acidity developed within the gland tubules and produced its destructive action there first. If the latter is true, the jejunal mucosa might become involved afterwards. The experiment in which the transplanted mucosa was involved might be interpreted in this way. The evidence tends to show that the acidity is but a secondary, although necessary factor and that the primary cause lies in the intrinsic mechanism of the gastric mucosa. However, the fact that changes in the jejunal mucosa occurred in one experiment shows the importance of acidity.

Protocols.

Dog 1.—Under ether anesthesia a jejunal transplant was made on June 29, 1915. The animal quickly recovered from the effects of the operation and its health was excellent. Killed Oct. 19.

Autopsy.—The anastomosis and transplant were in good condition. The transplant showed thickening of the muscularis mucosa as compared with the control part of the jejunum. The size of the transplant was 7.5 by 3 cm. The mucosa of the stomach and of the transplant was otherwise negative (Fig. 1).

Dog 2.—Under ether anesthesia a jejunal transplant was made on Oct. 14, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 11, the left adrenal was removed. The animal died from adrenal insufficiency on Apr. 21.

Autopsy.—The usual findings in animals dying from adrenalectomy were noted. The stomach was contracted and contained about 10 cc. of bile-stained, acid fluid. The gastric mucosa was injected throughout. In the pyloric region were four ulcers which measured 4 mm. in diameter, and probably extended to the muscularis mucosa. The transplant was 4 by 2.2 cm., and did not differ in any respect from the controls (Fig. 2).

Dog 3.—Under ether anesthesia a jejunal transplant was made on Oct. 12, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 16, the left adrenal was removed. The animal died from adrenal insufficiency on Apr. 21.

Autopsy.—The usual findings of adrenalectomized animals were noted. The stomach contained 10 cc. of bile-stained, acid fluid. The gastric mucosa was bile-stained and injected throughout. In the pyloric mucosa was one small ulcer. At the edge of the transplant in the gastric mucosa a large ulcer was found. The latter, however, may have been partially due to a retained suture. The transplant was 8 by 2.5 cm., and appeared in every respect similar to the controls (Fig. 3).

Dog 4.—Under ether anesthesia a jejunal transplant was made on Oct. 29, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 16, the left adrenal was removed. The animal died on Apr. 21 from adrenal insufficiency.

Autopsy.—The findings characteristic of an adrenalectomized animal were noted. The stomach contained a small amount of bile-stained, acid fluid. There were no frank gastric ulcers, but the mucosa was markedly injected and there were many areas of erosion. The transplant was 3 by 1.5 cm., and appeared similar to the controls.

Dog 5.—Under ether anesthesia a jejunal transplant was made on Oct. 13, 1915. The animal continued in excellent health. On Feb. 8, 1916, the right adrenal was removed. The animal remained in perfect condition. The left adrenal was removed on Apr. 24. The animal died from adrenal insufficiency on May 2.

Autopsy.—The usual findings in animals dying after adrenalectomy were noted. The stomach contained about 50 cc. of bile-stained, acid fluid. The fundal mucosa was markedly injected and contained many ulcers varying from 0.2 to 0.8 cm. in diameter. The pyloric mucosa showed a few ulcers. In the transplant were a few pitted areas which microscopically proved to be small ulcers. The size of the transplant was 3.5 by 3 cm. (Fig. 4).

SUMMARY.

Acute gastric ulcers are found in a large percentage of animals dying from acute adrenal insufficiency. In dogs in which a portion of the jejunum had been transplanted some time previous to the removal of the adrenals, the gastric mucosa showed more marked changes than the transplanted jejunal mucosa. This tends to show that the gastric juice as the cause of the ulcer is but a secondary although necessary factor.

EXPLANATION OF PLATES.

Plate 36.

Fig. 1. Gastric and jejunal mucosa of Dog 1. This shows the characteristic appearance of the transplant in normal dogs.

Fig. 2. Gastric mucosa and transplant of Dog 2. Note the ulcers in the pyloric mucosa and the normal appearance of the transplanted jejunal mucosa.

Plate 37.

Fig. 3. Gastric mucosa and transplant of Dog 3. The large ulceration is probably due to a retained silk suture. Note the injection of the gastric mucosa as compared with the jejunal transplant.

Fig. 4. Gastric mucosa and transplant of Dog 5. Note the marked injection, erosion, and ulceration of the gastric mucosa and the pin-point erosions of the transplanted jejunal mucosa.

AGGLUTINATION OF TREPONEMA PALLIDUM IN HUMAN SYPHILIS.

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In 1913 one of us (1) first described agglutination of a pure culture of *Treponema pallidum* by the sera of rabbits injected with a living and heat-killed culture.[1] Normal rabbit sera were found not to agglutinate this culture in dilutions as low as 1:20 (lower dilutions were not used), while the sera of immunized animals were found capable of agglutinating the culture in dilutions as high as 1:1,280. The agglutinating power of the sera of nineteen individuals in various stages of syphilis and of ten non-syphilitic individuals was studied in dilutions ranging from 1:20 to 1:640, but with negative results, due in large part, as we now believe, to the fact that lower dilutions of serum were not employed.

Previous to this, attempts had been made by Hoffmann and von Prowazek (2), Herxheimer and Löser (3), Hoffmann (4), Brönnum and Ellermann (5), Babes and Panea (6), Metchnikoff and Roux (7), Landsteiner and Mucha (8, 9), Zabolotny and Maslakowetz (10), and Uhlenhuth and Mulzer (11) to determine the presence of agglutinins for *Treponema pallidum* in the sera from human and experimental syphilis according to the grouping of the spirochetes in the tissues and with extracts and emulsions of tissues rich in the microparasites. While the presence of an agglutinin was suspected, technical difficulties, owing to the emulsion employed in the absence of pure cultures, did not permit the deduction of any positive conclusions.

In 1913, Nakano (12) found agglutinins for a culture of *Treponema pallidum* in the sera of rabbits injected intravenously with a heat-killed culture, that yielded well marked agglutination in dilutions from 1:10 to 1:70. Kissmeyer (13) working with a pure culture reported that normal human serum may agglutinate *Treponema pallidum* in dilutions as high as 1:50; he also tested the sera of 59 individuals in the various stages of syphilis and reported positive results in dilutions of 1:100 and higher in about 40 to 60 per cent of cases in the primary, secondary, and tertiary stages, including congenital syphilis, while the sera of immunized rabbits were reported as yielding well marked agglutination in dilutions

[1] The culture was obtained from Dr. Hideyo Noguchi of The Rockefeller Institute for Medical Research.

ranging from 1: 2,000 to 1: 200,000. Zinsser and Hopkins (14) have found that normal rabbit serum may agglutinate *Treponema pallidum* in dilutions of 1: 10 and lower, and the sera of immunized animals as high as 1: 2,000. They also stated that "normal human sera will agglutinate similar *pallidum* emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions," but gave no details of this portion of their work. In a second paper Zinsser, Hopkins, and McBurney (15) present important evidence indicating that the immune agglutinins in the sera of immunized rabbits and sheep failed to cause agglutination of virulent *pallida* from human lesions.

In this communication we desire to give the results of a study of agglutination of a pure culture of *Treponema pallidum* by normal human sera, the sera of persons in the various stages of syphilis, and the sera of non-syphilitic persons suffering with various other diseases.

Method.

Sera.—The sera of 12 persons known not to be syphilitic and with negative Wassermann reactions were studied; also the sera of 5 persons late in the primary, and 12 in the secondary stages of syphilis; 22 from patients with paresis; 12 from patients with positive Wassermann or luetin reactions and regarded as being tertiary or latent syphilitics; and 8 from persons with negative Wassermann reactions and denying syphilitic infection, but suffering with various other infections at the time when these tests were made.

Culture.—All the tests here reported were conducted with Zinsser's Strain A culture of *Treponema pallidum.* This culture was received growing under paraffin oil in ascitic broth over an egg medium and has been successfully grown in large tubes of egg medium covered with ascitic broth and paraffin oil. This method has proven very satisfactory, and luxuriant growths of active treponemata are secured uniformly within a 4 weeks' period of incubation.

After removal of the paraffin oil the fluid cultures in each tube were removed to sterile flasks with particular care not to break up the solid media and to remove the whitish sediment of treponemata which collects in the angle between the slant of egg media and the wall of the test-tube. After shaking the emulsions with sterile glass beads and centrifuging briefly, they were ready for use. Living cultures were employed throughout, and the emulsions were of such

density that each microscopic field examined by dark-field illumination showed from 12 to 30 treponemata.

Technique.—The macroscopic test was employed in exactly the same manner as in the previous study by Kolmer, except that lower dilutions were used; namely, 1: 2, 1: 5, 1: 10, 1: 20, 1: 40, and 1: 80.

1 cc. of treponema emulsion was used in each test, the serum being diluted in 1 cc. of sterile saline solution; the total volume, therefore, being 2 cc. with this amount of culture, a sufficient number of spirochetes were present to yield unmistakable agglutination in positive reactions. The first dilution (1: 2) was prepared by adding 1 cc. of emulsion to 1 cc. of serum; further dilutions of serum in 1 cc., as 1: 2.5, 1: 5, 1: 10, 1: 20, and 1: 40, were prepared, which after the addition of 1 cc. of emulsion were doubled in volume.

All the sera were unheated in dilutions varying from 1: 2 to 1: 80. After the preparations were set up the tubes were incubated for 2 hours at 37°C. and then placed in the refrigerator over night, final readings being made the next morning.

In every instance culture controls in normal saline solution were included; there were no evidences of spontaneous agglutination. As pointed out in the former communication, two or three spirochetes may be found in a loose clump, but in no instance were the large, clearly defined clump of spirochetes found, as in positive agglutinations.

In all tests a microscopic examination with the dark-field illuminator was made. Occasionally a light precipitate due to constituents in the culture medium may collect in the bottom of the small test-tubes, which macroscopically may be mistaken for agglutinated spirochetes; furthermore, the emulsions were not of sufficient density when diluted with an equal quantity of the serum dilution to render reliable a macroscopic reading of the reactions. For these reasons, we have examined each tube microscopically, removing the sediment or supernatant fluid or both with a fine capillary pipette and examining a drop by dark-field illumination.

In every instance the controls and negative reactions showed the presence of a large number of spirochetes, a few still motile, and all free from clumping. In a few instances two or three spirochetes were found in clumps, but there was no sedimentation and this was not

considered as an evidence of agglutination. In all positive reactions a drop of the sediment examined by dark-field illumination showed the presence of large clumps of spirochetes which were in most instances non-motile, although a few motile organisms were to be found at the edge of a clump, or entirely free. In the higher dilutions where agglutination was incomplete, free active spirochetes were found in the supernatant fluid and clumps in the sediment.

Wassermann Reactions.—All sera were submitted to the Wassermann reaction with three lipoidal antigens; namely, an alcoholic extract of human heart reenforced with cholesterol (C.H.), an alcoholic extract of syphilitic liver (S.), and an extract of acetone-insoluble lipoids from human heart muscle (A.).

With a portion of the sera an antigen of treponema in the form of luetin was employed; this antigen was titrated before each experiment and used in an amount equivalent to one-third of the anti-complementary dose.

All reactions were read immediately after the secondary period of incubation.

RESULTS.

Normal Human Sera.—The results observed with twelve sera are shown in Table I.

Half of these sera showed no evidence of agglutination in the lowest dilution; namely, equal parts of undiluted serum and emulsion. As pointed out above, however, we have not designated as agglutination the loose clumps of two or three spirochetes which are occasionally encountered in tests with normal sera and in the controls.

In 50 per cent of these sera there were some evidences of agglutination in the lowest dilution, but none in dilutions of 1: 5 and higher.

In our opinion agglutination of culture *Treponema pallidum* by human sera must occur in dilutions of 1: 5 or higher before any significance may be attached to the result from the standpoint of syphilis.

Sera of Non-Syphilitic Individuals Suffering with Other Infections.—The results observed with eight of these sera are shown in Table II and are similar to those observed with the sera of normal persons shown in Table I. Several of these patients had moderate fever at the time the tests were made, but this factor and the presence of

TABLE I.

Agglutination of Treponema pallidum by the Sera of Normal Individuals.

Case No.	Wassermann reaction.			Agglutination.					
	C. H.*	S.†	A.‡	1:2	1:5	1:10	1:20	1:40	1:80
1	−§	−	−	−	−	−	−	−	−
2	−	−	−	±	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−
4	−	−	−	+	−	−	−	−	−
5	−	−	−	±	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−
9	−	−	−	+	−	−	−	−	−
10	−	−	−	±	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−
12	−	−	−	±	−	−	−	−	−

* Cholesterolized alcoholic extract of human heart.

† Alcoholic extract of syphilitic liver.

‡ Acetone-insoluble lipoids of human heart.

§ − indicates absence of agglutination; resembles the salt solution controls. ± indicates slight but unmistakable agglutination characterized by numerous clumps of spirochetes; free spirochetes also occur in the supernatant fluid. + indicates well marked agglutination.

TABLE II.

Agglutination of Treponema pallidum by the Sera of Non-Syphilitic Individuals.

Case No.	Diagnosis.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Tuberculosis of penis	−	−	−	+	−	−	−	−	−
2	" " skin	−	−	−	−	−	−	−	−	−
3	" " lungs	−	−	−	−	−	−	−	−	−
4	Typhoid fever	−	−	−	±	−	−	−	−	−
5	Lobar pneumonia	−	−	−	±	−	−	−	−	−
6	Pyosalpingitis	−	−	−	−	−	−	−	−	−
7	Pregnancy	−	−	−	−	−	−	−	−	−
8	"	−	−	−	+	−	−	−	−	−

other antibodies in the sera, notably typhoid agglutinin, had no effect upon the spirochetes.

Primary Syphilis.—The results observed with five sera are shown in Table III.

TABLE III.

Agglutination of Treponema pallidum by the Sera of Patients in the Primary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++*	++	++	±	–	–	–	–	–
2	2 doses "	++++	++	+++	+	±	–	–	–	–
3	1 dose "	++	–	–	+	±	–	–	–	–
4	None..................	+	–	–	–	–	–	–	–	–
5	"	++	–	+	±	–	–	–	–	–

* ++++ indicates complete inhibition of hemolysis (strongly positive); +++, 75 per cent inhibition of hemolysis (moderately positive); ++, 50 per cent inhibition of hemolysis (weakly positive); +, 25 per cent inhibition of hemolysis (very weakly positive); ±, 10 per cent inhibition of hemolysis (doubtfully positive).

The first three patients were tested late in the primary stage and after treatment with arsenobenzol had been instituted; Cases 4 and 5 were tested about 2 weeks after the appearance of the chancre and before the treatment was begun.

In all cases the Wassermann reaction was positive; in Nos. 3 and 4 the reaction was positive with the cholesterolized extract only.

While we cannot draw conclusions from the examination of so few cases in this stage of syphilis, it would appear that traces of agglutinin for *Treponema pallidum* may be found in the serum in certain cases late in the primary stage. This opinion is based upon the higher percentage of partial agglutinations in the 1:2 dilution as compared with the sera of normal persons, and upon the occurrence of partial agglutination in the 1:5 dilution in two of the five sera.

Secondary Syphilis.—The results observed with the sera of twelve persons are shown in Table IV.

TABLE IV.

Agglutination of Treponema pallidum by the Sera of Patients in the Secondary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++	++	++	+	+	±	–	–	–
2	1 " "	+	–	–	+	–	–	–	–	–
3	None.....................	++++	++++	++++	+	±	–	–	–	–
4	2 doses arsenobenzol	++++	++++	++++	±	–	–	–	–	–
5	2 " "	++++	++	++++	–	–	–	–	–	–
6	2 " "	++++	+++	++++	+	+	+	+	–	–
7	Mixed (2 wks.).............	++++	++++	++++	+	±	–	–	–	–
8	None.....................	++++	++++	++++	+	–	–	–	–	–
9	6 doses arsenobenzol	–	–	–	–	–	–	–	–	–
10	4 " "	+++	++	++	+	±	–	–	–	–
11	None.....................	++++	++++	++++	+	+	–	–	–	–
12	"	++++	++++	++++	+	+	±	–	–	–

All but four of these patients were tested after treatment had been instituted; all but one (Case 9) gave positive Wassermann reactions.

The sera of seven patients showed the presence of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination found in dilutions higher than 1:20.

These results appear to show quite definitely that agglutinins for *Treponema pallidum* are present in the sera of a proportion of cases of syphilis in the secondary stage.

Tertiary Syphilis.—The results observed with the sera of thirty-seven persons regarded as being in the tertiary or late latent stages of syphilis are shown in Tables V and VI.

In Table V are shown the results of agglutination tests with the sera of twenty-one persons regarded clinically as being well developed cases of paresis and of one person in whom the clinical diagnosis was doubtful paresis.

All but one of these sera agglutinated *Treponema pallidum*, and in the majority of instances in dilutions of 1:5 and higher. One serum caused well marked agglutination in dilutions as high as 1:80; the majority showed agglutination in dilutions varying from 1:5 to 1:40.

TABLE V.

Agglutination of Treponema pallidum by the Sera of Patients Suffering with Paresis.

Case No.	Diagnosis.	Wassermann reaction.				Agglutination.					
		Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Paresis............	−	+++−	+	++++	+	+	+	+	−	−
2	"	±	++++	++	++++	+	−	−	−	−	−
3	"	±	++++	±	++++	+	+	+	−	−	−
4	"	±	++++	++++	++++	+	+	−	−	−	−
5	"	−	++++	++++	++++	+	+	+	−	−	−
6	"	−	++++	+	++	+	+	+	+	+	−
7	"	−	++++	++++	++++	+	+	+	+	−	−
8	"	+	+++	−	−	+	+	+	+	+	−
9	"	−	+	+	+	+	+	+	+	+	−
10	"	+	++++	++++	++++	+	+	+	+	+	+
11	"	+	++++	++++	++++	+	+	+	−	−	−
12	"	−	±	−	−	+	+	+	+	−	−
13	"	−	++++	++++	++++	+	+	+	−	−	−
14	"	−	+++	+++	+++	+	+	+	−	−	−
15	"	−	++	++	++	−	−	−	−	−	−
16	"	+	++++	++++	++++	+	±	−	−	−	−
17	"	+	++++	++++	++++	+	+	+	+	+	−
18	"	−	++++	++++	++++	+	+	±	−	−	−
19	"	−	++++	++++	++++	+	+	+	−	−	−
20	"	−	++++	++++	++++	+	+	+	−	−	−
21	"	+	++++	++++	++++	+	+	+	+	+	−
22	" (doubtful)...	−	−	−	−	+	+	±	−	−	−

In Table VI are shown the results observed with a group of cases yielding positive Wassermann or luetin reactions or both and regarded clinically as being syphilitic.

None of the five cases regarded clinically as dementia præcox of different types gave positive Wassermann reactions, although all these cases yielded a papular or pustular luetin reaction, and in three of them there was well marked agglutination of *Treponema pallidum.*

The results of these agglutination tests with the sera of patients in the late stages of syphilis show that agglutinin is especially likely to be found in the highest percentages of cases and in largest amounts at this period of the disease.

TABLE VI.

Agglutination of Treponema pallidum by the Sera of Patients in the Tertiary Stage of Syphilis.

Case No.	Diagnosis.	Luetin skin test.*	Wassermann reaction.				Agglutination.					
			Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Tabes dorsalis	++	–	++	–	–	+	+	+	+	–	–
2	Taboparesis	+	–	++++	++++	++++	+	–	–	–	–	–
3	"	+	–	++++	++++	++++	+	+	+	–	–	–
4	Cerebrospinal syphilis	++	++	++++	++++	++++	+	+	+	+	–	–
5	" "	±	–	++++	++++	++++	+	–	–	–	–	–
6	Dementia præcox	++	–	–	–	–	+	+	±	–	–	–
7	" "	+	–	–	–	–	+	+	+	–	–	–
8	" "	+	–	–	–	–	±	–	–	–	–	–
9	" "	++	–	–	–	–	+	+	+	+	+	+
10	" "	+	–	–	–	–	±	–	–	–	–	–
11	Depressive mania	+	–	+	–	–	+	+	+	+	–	–
12	" "	+	–	++	–	–	+	+	–	–	–	–
13	Aortic disease	0	+	++++	++++	++++	+	+	+	+	–	–
14	" aneurysm	0	–	++++	+	++	+	+	+	–	–	–
15	Gumma of brain	0	–	++++	++	+++	+	+	+	+	+	–

* ++ indicates pustular reaction; +, well defined papular reaction; ±, slight papular reaction.

Agglutination and Complement Fixation.

An analysis of the results of agglutination and complement fixation tests with the sera of 54 persons yielding positive Wassermann or luetin reactions or both and regarded clinically as syphilitic, shows the following:

1. The Wassermann antibody seems to appear in the blood serum before demonstrable amounts of agglutinin.

2. The Wassermann antibody appears to reach a much higher degree of concentration in the blood serum.

3. The Wassermann antibody may be present while sufficient agglutinin to produce agglutination in dilutions of 1:5 and higher is absent, dilutions lower than 1:5 being unreliable because of the possibility of agglutination in a dilution of 1:2 by the sera of normal persons. Of the 54 sera from all stages of syphilis yielding positive

Wassermann reactions agglutination in dilutions of 1:5 and higher was absent in 14 sera or 26 per cent.

4. On the other hand, in 4 sera, or about 7 per cent of this series, demonstrable amounts of agglutinin were present and the Wassermann antibody was absent.

5. Of 37 patients regarded as being in the later stages of syphilis (Tables V and VI), 31 yielded positive Wassermann reactions and 8 positive and 3 doubtfully positive reactions with an antigen of luetin; all the sera yielding positive reactions with the antigen of luetin showed the presence of a demonstrable amount of agglutinin; on the other hand, a number of sera showing the presence of agglutinin did not absorb complement with the luetin antigen.

SUMMARY.

The results observed with this strain of *Treponema pallidum* may be stated as follows:

1. Normal human serum did not agglutinate this culture of *Treponema pallidum* in dilutions of 1:5 or higher; equal parts of treponema culture and normal serum (dilution 1:2) resulted in agglutination in about 50 per cent of the sera. With the strain of *Treponema pallidum* used in this study specific agglutination was not considered as having occurred unless observed in dilutions of 1:5 or higher.

2. The agglutinating power of the sera of non-syphilitic persons suffering with various infections was not higher than that of normal sera.

3. Traces of specific agglutinin for this culture of *Treponema pallidum* were found in the sera of persons late in the primary stage of syphilis.

4. In the secondary stage of syphilis about 58 per cent of sera showed the presence of a demonstrable amount of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination in evidence in dilutions higher than 1:20.

5. In tertiary and latent syphilis, mostly infections of the central nervous system, agglutination in dilutions of 1:5 and higher was found with about 84 per cent of sera. The highest concentration of *pallida* agglutinin in the blood serum was found in the later stages of syphilis.

6. The formation of agglutinin for culture *Treponema pallidum* in human syphilis is probably tardy and does not reach a state of high concentration.

7. There was no direct relation between the presence of the Wassermann antibody and agglutinin for this strain in the sera of syphilitics. The former appears in the body fluids earlier and is probably in higher concentration in all stages of syphilis; while agglutination is most likely to occur with Wassermann positive sera, it may be occasionally observed with sera yielding negative Wassermann reactions. Likewise strongly Wassermann positive sera may not contain demonstrable amounts of agglutinin.

8. It is probable that different strains of *pallida* vary in susceptibility to agglutination. For example, in the first investigation on agglutinins for *Treponema pallidum* by Kolmer, the sera of syphilitics in different stages of the disease did not cause agglutination of one of Noguchi's strains in dilutions of 1:20 and higher, whereas the strain used in the present study was frequently agglutinated in this dilution by sera from a similar group of patients. As shown by Zinsser, Hopkins, and McBurney, virulent *pallida* from human lesions resist agglutination to a remarkable degree.

9. While further studies are necessary to establish the practical value of agglutination in the diagnosis of human syphilis, we believe that with a suitable culture or cultures well marked agglutination of *Treponema pallidum* in dilutions of 1:5, or better 1:10 and higher, indicates *pallida* infection and may prove of value in the diagnosis of isolated cases, particularly in the later stages. Investigations bearing upon the relation of agglutinins for culture *pallida* to immunity in syphilis will be given in a separate communication (16).

BIBLIOGRAPHY.

1. Kolmer, J. A., Concerning Agglutinins for *Treponema pallidum*, *J. Exp. Med.*, 1913, xviii, 18–24.
2. Hoffmann and von Prowazek, cited by Neisser and Bruck in Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911, 205; also *Arb. k. Gsndhtsamte.*, 1911, xxxvii, 205.
3. Herxheimer, K., and Löser, Ueber den Bau der *Spirochaete pallida*, *Münch. med. Woch.*, 1905, lii, 2212–2213.

4. Hoffmann, E., Mitteilungen und Demonstrationen über experimentelle Syphilis, *Spirochaete pallid* und andere Spirochaetenarten, *Dermat. Z.*, 1906, xiii, 561–565.
5. Brönnum, A., and Ellermann, V., *Spirochaete pallida* in den inneren Organen bei Syphilis hereditaria, *Deutsch. med. Woch.*, 1905, xxxi, 1757–1758.
6. Babes, V., and Panea, J., Ueber pathologische Veränderungen und *Spirochaete pallida* bei congenitaler Syphilis, *Berl. klin. Woch.*, 1905, xlii, 865–869.
7. Metchnikoff, E., and Roux, E., Etudes expérimentales sur la syphilis, *Ann. Inst. Pasteur*, 1905, xix, 673–698.
8. Landsteiner, K., and Mucha, V., Beobachtungen über *Spirochaete pallida*, *Centr. Bakteriol., 1te Abt., Ref.*, 1907, xxxix, 540–541.
9. Landsteiner and Mucha, Zur Technik der Spirochaetenuntersuchung, *Wien. klin. Woch.*, 1906, xix, 1349–1350.
10. Zabolotny, D., and Maslakowetz, Beobachtungen über Beweglichkeit und Agglutination der *Spirochaete pallida*, *Centr. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532–534.
11. Uhlenhuth, P., and Mulzer, P., Beiträge zur experimentellen Pathologie und Therapie der Syphilis mit besonderer Berücksichtigung der Impf-Syphilis der Kaninchen, *Arb. k. Gsndhtsamte.*, 1913, xliv, 307–530.
12. Nakano, H., Über Immunisierungsversuche mit Spirochaeten-Reinkulturen, *Arch. Dermat. u. Syph.*, 1913, cxvi, 265–280.
13. Kissmeyer, A., Agglutination der *Spirochaete pallida*, *Deutsch. med. Woch.*, 1915, xli, 306–308.
14. Zinsser, H., and Hopkins, J. G., Antibody Formation against *Treponema pallidum*—Agglutination, *J. Exp. Med.*, 1915, xxi, 576–583.
15. Zinsser, H., Hopkins, J. G., and McBurney, M., Studies on *Treponema pallidum* and Syphilis. IV. The Difference in Behavior in Immune Serum between Cultivated Non-Virulent *Treponema pallidum* and Virulent Treponemata from Lesions, *J. Exp. Med.*, 1916, xxiii, 341–352.
16. Kolmer, J. A., and Broadwell, S., Jr., The Relation of the Luetin Skin Reaction to Immunity in Syphilis. The Treponemicidal Action of the Serum in Human Syphilis, *J. Immunol.* (in press).

A STUDY OF THE PHYSIOLOGICAL ACTIVITY OF ADENOMATA OF THE THYROID GLAND, IN RELATION TO THEIR IODINE CONTENT, AS EVIDENCED BY FEEDING EXPERIMENTS ON TADPOLES.

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Plates 38 to 40.

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In 1912 and 1914 Gudernatsch[1] published the results of his experiments on feeding thyroid and other animal tissues to tadpoles. He concluded that thyroid has the "power to excite differentiation, but it lacks the power to cause growth." He used for these experiments fresh thyroid glands, the iodine content of which was not determined.

In 1914 Lenhart[2] carried out experiments along the same line as regards the thyroid, using for this purpose desiccated human, canine, sheep, and ox thyroids with iodine determinations on each specimen. He states that "the feeding of dried thyroid gland to tadpoles causes an early differentiation in proportion to the quantity fed or the percentage of iodine content of the gland used," and that thyroids with sufficiently low iodine content caused earlier differentiation but did not interfere materially with growth. "It all seems a question of dosage."

In Lenhart's work non-tumorous thyroid tissue was used. Since it has been established that the action of non-tumorous thyroid on tadpoles is dependent upon the iodine content, the following study was made to determine whether or not the so called tumors (adeno-

[1] Gudernatsch, J. F., Feeding Experiments on Tadpoles. I. The Influence of Specific Organs Given as Food on Growth and Differentiation, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 457; Feeding Experiments on Tadpoles. II. A Further Contribution to the Knowledge of Organs with Internal Secretion, *Am. J. Anat.*, 1913–14, xv, 431.

[2] Lenhart, C. H., The Influence upon Tadpoles of Feeding Desiccated Thyroid Gland in Variable Amounts and of Variable Iodine Contents, *J. Exp. Med.*, 1915, xxii, 739.

mata) of the thyroid, including carcinoma, wou'd have the same action as non-tumorous thyroid and whether this action corresponds to the iodine content of the tumors.

For this purpose tadpoles (*Rana pipiens* and *Rana clamata*) were brought to the laboratory on May 8, 1915. They were fairly uniform in size (10 to 13 mm.) and age (estimated at about 1 week). The stock was kept in the laboratory in large granite basins and fed on fresh liver every day, the water (city tap) being changed once each day.

Thyroid Preparations.—Human thyroids were used in the experiments (Table I). Twenty-one specimens of desiccated thyroid were prepared from eighteen glands removed in Dr. Crile's clinic at Lakeside Hospital. Except in the case of No. 16 the portions of the fresh gland to be used were chopped fine, placed in a drying oven at 70°C. within 1 hour after removal, and allowed to remain in the desiccator from 4 to 6 days; they were then ground to a fine powder in a mortar and kept in sterile bottles until used. Iodine determinations were done on each specimen.[3] No. 16 was prepared from the same gland as No. 15 after the latter had been fixed in formalin. This was done to determine the effect of formalin fixation on the iodine content and the action on tadpoles.

The list of thyroids includes two simple adolescent colloid goiters (Nos. 12, 15, and 16) without tumors or complications; one unclassified gland (No. 3) which probably represents a stage between simple colloid goiter and diffuse colloid adenomatous goiter; and three diffuse colloid adenomatous goiters (Nos. 1, 2, 6, and 17) in which there was a diffuse colloid adenomatous change throughout the whole gland. There were also eleven glands with well encapsulated single or multiple adenomata of the fetal series in various stages of growth and differentiation from the almost pure fetal type to the well differentiated colloid or simple adenoma. Many of these showed some or all of the secondary degenerative changes frequently occurring in these tumors; namely, edema, recent and old hemorrhage, hyaline scars, and areas of calcification and cyst formation. This group includes in the order of their increasing differentiation Nos. 21, 19, 8, 4, 13, 20, 9, 10, 18,

[3] The iodine determinations were done by Dr. Marine.

TABLE I.

Desiccated Thyroid Preparations.

Thyroid No.	Specimen No.	Age.	Duration of goiter.	Clinical diagnosis.	Weight of specimen.	Pathological diagnosis.	Iodine per gm. of dried gland.
		yrs.	*yrs.*		*gm.*		*mg.*
1	11,714	57	20	Goiter, adenomatous.	700	Diffuse colloid adenomatous goiter.	0.22
2	11,714	57	20	" "	700	Diffuse colloid adenomatous goiter.	0.12
3	11,725	26	12	" " colloid, multiple.	735	Unclassified.	0.55
4	11,765	29	16	Goiter, adenomatous.	70	Hyperplastic intermediate adenoma.	0.69
5	11,818	30	18	" "	200	Intermediate adenoma, well differentiated.	1.31
6	11,819	55	31	" colloid.	225	Diffuse colloid adenomatous goiter.	0.43
7	11,821	34	7	" adenomatous.	173	Multiple intermediate adenoma, well differentiated.	0.85
8	11,825	47	15	" "	145	Intermediate adenoma.	0.06
9	11,836	44	14	" "	90	Multiple intermediate adenoma.	0.00 or trace.
10	11,836	44	14	" "	90	Multiple intermediate adenoma.	0.15
11	11,844	61	12	" multiple.	70	Multiple intermediate adenoma, well differentiated.	0.17
12	11,878	21	6	" colloid.	192	Colloid goiter, adolescent type.	0.58
13	11,886	47	40	" adenomatous.	14	Multiple intermediate adenoma.	1.23
14	11,898	58	$1\frac{1}{2}$	Adenoma.	273	Carcinoma; malignant adenoma.	0.00 or trace.
15	11,904	18	2	Colloid goiter. Appendicitis.	87	Colloid goiter, adolescent type.	1.00
16	11,904	18	2	Colloid goiter. Appendicitis.	87	Colloid goiter, adolescent type. (Formalin fixation.)	0.85
17	11,932	29	20	Colloid goiter.	710	Diffuse colloid adenomatous goiter.	0.18
18	11,933	55	12	Goiter, adenomatous.	545	Degenerating cystic intermediate adenoma.	0.07
19	11,938	31	16	" "	40	Intermediate adenoma.	0.06
20	11,939	44	$\frac{1}{3}$	" exophthalmic.	75	Multiple intermediate adenoma, well differentiated.	4.31
21	11,944	27	9	" adenomatous.	250	Fetal adenoma.	0.00 or trace

11, 7, and 5. There was one carcinoma (No. 14) of the malignant adenoma type.

In Nos. 1 and 2, 9 and 10, and 15 and 16, two preparations were made from each of three different glands. No. 20 was diagnosed as exophthalmic goiter, and No. 13 had been treated in the clinic 1 year previously for exophthalmic goiter. The others were diagnosed as simple colloid goiter or simple adenomatous goiter before operation. The carcinoma was not diagnosed as a malignant tumor before operation.

In the preparation of the specimens of adenomata the tumorous tissue was stripped from its capsule, care being taken not to include any of the surrounding non-tumorous thyroid tissue. In the case of the diffuse colloid adenomatous goiters, individual adenomatous nodules were shelled out with their capsules and the total mass was treated as described.

EXPERIMENTAL.

Series A.—May 11 to June 10, 1915 (duration, 31 days). The tadpoles were kept on a table in the center of the room so that light and temperature conditions were the same for all. Temperatures of the room and water were recorded each day.

Five tadpoles were placed in each of twenty-one granite dishes of about 500 cc. capacity, using 300 cc. of city tap water. Four basins with five tadpoles each were used as controls. Sample tadpoles were killed in formalin at the beginning of the experiment for standards of comparison. The tadpoles for experiment were fed 50 mg. of desiccated thyroids, Nos. 1 to 21, every 2nd day, alternating with fresh liver. The controls were fed fresh liver every 2nd day. The tadpoles in this series were not so uniform in size as was desired.

For this series merely the date of death in the different dishes may be given. The action of the different specimens of thyroid was essentially the same as to emaciation, growth, and differentiation as in Series B which is to be described in detail and will serve for both.

The following tabulation in conjunction with Fig. 1 gives a fair idea as to the time of death of the tadpoles and their condition at the time of death or at the termination of the experiment when they were killed in formalin.

Thyroid No.	Iodine.	Result.	Average time of death.
	mg.		days
20	4.31	All dead in 14 days.	12.4
5	1.31	" " " 16 "	13.8
13	1.23	" " " 21 "	12.8
15	1.00	" " " 22 "	21
16	0.85	" " " 24 "	22
7	0.85	" " " 19 "	16
4	0.69	" " " 28 "	23
12	0.58	" " " 24 "	22.4
3	0.55	Two " " 26 "; three lived.	31
6	0.43	All " " 29 "	22.8
1	0.22	All alive at end of 31 days.	
17	0.18	One dead in 25 days; four lived.	31
11	0.17	Two " " 29 "; three "	31
10	0.15	All alive at end of 31 days.	
2	0.12		
18	0.07		
8	0.06		
19	0.06	One dead in 30 days; four lived.	31
21	0.00	All but one, No. 14, lived 31 days.	
14	0.00		
9	0.00		

Controls. Sixteen lived 31 days; no forelegs present. Four died showing no other differentiation than slight growth of the posterior leg buds.

Definite changes were first noticed in this series on the 6th day in Nos. 20, 5, 13, 15, and 12. There was some wasting, beginning atrophy of the tail, and increased growth of the posterior leg buds. At the time of death all these, as well as Nos. 16, 6, 4, and 7, were smaller than the samples or the controls and showed much more differentiation than the controls.

As to the appearance of forelegs it is noteworthy that in the tadpoles in No. 20, four of which died in 12 days, all had visible left foreleg buds. In No. 5 all died between the 14th and 16th days and all showed foreleg buds. In No. 13 two died in 10 days with foreleg buds scarcely visible; forelegs were present on the other three. In No. 15 all died between the 20th and 22nd days; foreleg buds were present on two and doubtful on the other three.

Others in this series developed forelegs at different times corresponding closely in this and other respects with Series B. It is interesting to point out, however, that the large percentage of tadpoles living 31

days were being fed on thyroids with iodine contents of 0.22 mg. or less. It is also interesting that all these latter grew as much or more than the controls, and most of them showed a greater degree of differentiation.

Series B.—May 11 to June 28, 1915 (duration, 49 days). The procedure was the same as that in Series A, but smaller, white porcelain dishes with 150 cc. of water were used. The tadpoles in this series were quite uniform in size.

Table II and Fig. 2 give the results in this series, using the time of appearance of the first foreleg as an index to differentiation. This we think is justifiable because with the most active thyroid preparations used, and even in those tadpoles dying as early as 8 days this evidence of differentiation was present. It also offers a convenient

TABLE II.

Series B, Arranged According to Iodine Content.

Thyroid No.	Iodine content.	Time of appearance of first foreleg.	Average time of appearance of first foreleg.	Average time of death or killing.
	mg.	*days*	*days*	*days*
20	4.31	12	12.8	12.8
5	1.31	12	12.6	12.4
13	1.23	14	17.2	17.2
15	1.00	16	21.7	23.2
16	0.85	21	25.6	25.0
7	0.85	22	23.6	24.4
4	0.69	8	19.6	20.6
12	0.58	25	25.0	28.4
3	0.55	25	32.0	38.4
6	0.43	31	37.8	39.4
1	0.22	34	42.2	45.2
17	0.18	38	40.0	42.0
11	0.17	34	43.7	42.8
10	0.15	38	40.0	45.0
2	0.12	41	41.0	29.0
18	0.07	36	38.0	33.0
8	0.06	40	44.5	47.0
19	0.06	40	41.6	39.4
21	0.00 or trace.	40	46.4	48.2
14	0.00 " "	37	45.6	46.4
9	0.00 " "	40	43.2	45.0
Controls....................		45		

and useful method of comparison in determining the action of desiccated thyroid in causing differentiation of tadpoles. This is usually a fairly decisive indicator and may be readily recognized within a period of 24 hours of its occurrence.

In comparing Series A and B it is interesting to note that in Nos. 20, 5, 13, 15, 16, 7, 4, and 12, with iodine contents varying from 4.31 to 0.58 mg., all the tadpoles were dead in each series at the end of 31 days, the duration of Series A. In No. 3 (iodine content, 0.55

TABLE III.

Series B, Arranged According to Effects Observed on the 14th Day of the Experiment.

Thyroid No.	Iodine content.	Time of appearance of first foreleg.	Average time of appearance of first foreleg.	Average time of death or killing.
	mg.	*days*	*days*	*days*
20 5 13	4.31–1.23	12–14	12.6–17.2	12.4–17.2
15	1.00	16	21.7	23.2
16 7 4 12 3 6	0.85–0.43	21–31 (No. 4 in 8 days.)	19.6–37.8	24.4–39.4 (No. 4 in 20.6 days.)
1 17 11 10 2	0.22–0.12	34–41	40–43.7	42–45.2 (No. 2 in 20 days.)
18 8 19	0.07–0.06	36–40	38–44.5	33–47
21 14 9	Trace to 0.00	37–40	43.2–46.4	45–48.2
Controls		45		

mg.), two tadpoles in Series A and one in Series B were dead. In No. 6 (iodine content, 0.43 mg.) all were dead in Series A but only one in Series B. Below this level of iodine content (0.22 to 0.00 or trace) 91 per cent of each series were still living at the end of 31 days. It is evident then that a detailed description of the results in Series B will suffice for both within the time limits of 31 days. Series B is chosen for detailed study because of its longer duration (49 days).

The action of the desiccated thyroid was striking and consistent. On the 14th day of the experiment, before the iodine contents of the different thyroid preparations had been determined, the tadpoles were grouped according to the effect and the relative iodine contents of the different preparations predicted upon this basis. The prediction was correct with iodine contents from 4.31 down to 0.43 mg. in the order given in Table III. Below the level of 0.22 individual distinctions could not be made out with certainty, but all in this group were less affected than those receiving specimens with higher iodine contents.

Protocols.

Thyroid 20.—Changes were first observed in this dish on the 5th day; that is, after the second dose of thyroid. The change consisted of beginning kite-shaped appearance, prominence of the head, diminution in size or wasting, beginning atrophy of the tail, and increased growth of the hind leg buds. Three of these died on the 12th day and two on the 14th. All showed the characteristic changes and all at the time of death had visible left foreleg buds; all were smaller than the samples or controls. One became edematous.

Thyroid 5.—The changes, first noticed on the 5th day, were the same as in No. 20. Four died on the 12th day and one on the 14th, showing marked wasting and tail atrophy; three had a frog-shaped body and two were definitely kite-shaped. At the time of death left foreleg buds were visible on three and not observed on two; all were smaller than the samples or controls; two were somewhat edematous.

Thyroid 13.—The changes, first observed on the 6th day, were similar to those in Nos. 20 and 5. One died on the 14th day, two on the 16th, and two on the 20th. The left foreleg was present on each. One dying on the 20th day had both foreleg buds. All were smaller than the samples or controls; four were somewhat edematous.

Thyroid 15.—Changes similar to the above were observed on the 6th day. All showed marked wasting, tail atrophy, and considerable differentiation. These died on the 16th, 23rd, 24th, 25th, and 28th days, respectively. Two had left foreleg buds present at the time of death; the one dying on the 28th day had

both forelegs and the body was beginning to present the typical frog shape. One in this dish was lost.

Thyroid 16.—Changes were first observed on the 7th day. One died on the 21st day, 2 in 24 days, and 2 in 28 days. The left foreleg was present on three; not observed on two. All in this dish were quite edematous; all showed typical head changes and some were assuming frog-shaped bodies.

Thyroid 7.—Changes were first observed on the 7th day. Three died in 22 days, and two in 28 days. The left foreleg bud was present on all; all showed typical changes and were assuming frog bodies; slight edema in all.

Thyroid 4.—Slight change of shape was first observed on the 7th day. These tadpoles died in 8, 22, 23, 24, and 26 days. All were smaller than the controls; four were edematous. They showed various degrees of tail atrophy and were slightly kite-shaped. The three dying in 8, 24, and 26 days, respectively, had left foreleg buds. None were observed on the other two.

Thyroid 12.—Slight change of shape was first observed on the 7th day. One died in 26 days, two in 28, and two in 30. The first was highly edematous and showed no differentiation beyond the controls; no foreleg bud. The others were small and had frog-like bodies; the left foreleg bud was present on each; tail atrophy one-half to two-thirds.

Thyroid 3.—A slight change of shape was first observed on the 8th day. One died in 28 days, one in 36, two in 40, and one in 48. The first had a tadpole body, no tail atrophy, posterior leg buds about the same as the controls and no forelegs, and was highly edematous. The second had slight tail atrophy and the left foreleg bud through the skin; it was smaller than the control. The other three had frog-like bodies; both forelegs were present on each; tail atrophy one-third to two-thirds.

Thyroid 6.—In this dish it was doubtful whether definite changes could be made out on the 8th day. The tadpoles died in 31, 36, 39, 42, and 49 days. At the time of death all had left forelegs; the third and fourth had both forelegs, and on the fifth the right foreleg was present under the skin. Three had typical frog-shaped bodies and two were slightly frog-shaped.

Thyroid 1.—No change was observed up to the 8th day. Four died in 40, 43, 45, and 49 days, respectively. All were well differentiated frogs with almost complete atrophy of the tail. One which lived 49 days had a tadpole body and no forelegs. This is the first tadpole of the series fed on thyroid with iodine of 0.22 mg. or above which lived the 49 days of the experiment. Above this level of iodine content the tadpoles showed progressive degrees of wasting, and early differentiation in proportion to the iodine content. In none of these did the thyroid-fed tadpoles keep up with the controls as to growth (size), but of course they showed greater differentiation. Below this level of iodine content the thyroid-fed tadpoles grew as well as, and in many cases better than the controls (growth inversely proportional to the iodine content). At the same time there was a greater degree of differentiation among the low iodine-fed tadpoles as compared with the controls.

Thyroid 17.—In the frogs in this dish forelegs appeared in 38, 39, 40, 41, and 42 days. The first four developed into normal frogs with well developed and functioning fore- and hind legs and were killed in formalin on the 41st day. The last one developed into a normal frog and was killed on the 46th day.

Thyroid 11.—One died in 34 days, with beginning atrophy of the tail, slight changes about the head, and no forelegs. This one was smaller than the controls. The other four developed forelegs in 34, 39, 46, and 46 days, respectively. Two of these were killed in 42 days, and the last two died on the 48th day. All four were well developed frogs.

Thyroid 10.—Forelegs appeared in 38, 39, and 43 days on three. These developed into normal frogs; one died on the 40th day and two were killed on the 43rd and 46th days. The other two became highly edematous, showed no greater differentiation than the controls, and were killed on the 48th day.

Thyroid 2.—In this dish the tadpoles died in 11, 22, 34, 37, and 41 days. The first three maintained the tadpole body, had no atrophy of the tail, and developed no forelegs. Some of these were counted accidental deaths and not attributed to thyroid action. The fourth, dead on the 37th day, was becoming frog-shaped; no tail atrophy; no forelegs. The fifth was the only one that developed a foreleg (41st day). This one had a well developed frog-shaped body; tail atrophy about one-half.

Thyroid 18.—One of these was lost; two died in 15 and 35 days; and two lived 41 days and were killed in formalin. The first (15 days) showed little or no differentiation beyond the controls; no forelegs. The second (35 days) had well developed hind legs and was assuming a frog-shaped body; no forelegs or tail atrophy. The third and fourth developed forelegs in 36 and 40 days, respectively, and were killed on the 40th day. One of these was a well developed frog with almost complete atrophy of the tail; the other developed good fore- and hind legs, with little atrophy of the tail, and was highly edematous.

Thyroid 8.—Four developed forelegs in 40, 42, 48, and 48 days, respectively. One, which lived 49 days, had a tadpole body, no tail atrophy, and no forelegs. The first three developed into normal frogs with functioning fore- and hind legs. The fourth had both forelegs but a tadpole body and little atrophy of the tail. The fourth and fifth, both having a tadpole body, were larger than the controls.

Thyroid 19.—Two died in 28 and 35 days, respectively, without forelegs. Tail atrophy had begun, one having a tadpole body, the other becoming frog-shaped. Two developed forelegs in 40 days, became normal frogs, and were killed on the 43rd day. The last one developed forelegs on the 45th day, and when killed on the 48th day was fairly well differentiated.

Thyroid 21.—Forelegs appeared in 40, 46, 48, 49, and 49 days, respectively. The first developed into a normal frog and was killed on the 45th day. The other four lived 49 days; two of these had well developed frog-shaped bodies, tail atrophy one-third, and functioning fore- and hind legs; one had the left foreleg; and the last one had the left foreleg under the skin.

Thyroid 14.—Forelegs appeared in 37, 45, 48, 49, and 49 days, respectively. The first died in 37 days, with a tadpole body, slight changes about the head, and the left foreleg under the skin; it was about the size of the controls. The second developed into a normal frog with functioning fore- and hind legs. The third had a well developed frog body, tail atrophy one-third, and the left foreleg present. The fourth and fifth were becoming frog-shaped, had slight tail atrophy, and left forelegs under the skin.

Thyroid 9.—Four developed forelegs in 40, 40, 45, and 48 days, respectively. These were well differentiated, had functioning fore- and hind legs, and various degrees of tail atrophy. The fifth was killed on the 43rd day; highly edematous.

Controls.—There were four dishes with five tadpoles in each. These received fresh liver every 2nd day. The first one developed the left foreleg on the 45th day and died on the 48th day, a well differentiated frog. On the 49th day there were four dead, one with well developed hind legs and a left foreleg, also beginning tail atrophy; one with well developed hind legs and no forelegs; the other two were well preserved tadpoles with short posterior leg buds, no forelegs, and no tail atrophy. Fifteen lived for 49 days and were well preserved tadpoles.

Fig. 2 shows the condition of the tadpoles in this series at the time of death or termination of the experiment when they were killed in formalin. The different groups are arranged according to the decreasing iodine content with the controls and samples last. The individuals of each dish are arranged from left to right according to the time of death; *e.g.*, in No. 20 the first three from left to right died in 12 days and the fourth and fifth died in 14 days.

We have no satisfactory explanation for the peculiar edematous appearance of some of the tadpoles. This was observed in individuals in different dishes without regard to the iodine content and appeared in some of the controls.

Series C and D were for the purpose of determining the action of desiccated thyroid on a different variety of tadpoles and at varying ages of this species (*Rana catesbiana*).

Series C.—May 25 to June 10, 1915 (duration, 17 days). (Fig. 3.)

Tadpoles averaging 4 cm. in length were used in this series. Three were killed in formalin at the beginning of the experiment for comparison; three were used as controls and fed fresh liver every 2nd day; the experimental tadpoles were fed 50 mg. of Thyroids 20, 15, 12, 17, and 14 with iodine contents of 4.31, 1.0, 0.58, 0.18, and 0.0, or trace, every 2nd day, alternating with fresh liver. At the beginning of the experiment the tadpoles had an average length of 4 cm., just visible posterior leg buds, tadpole bodies, and no sign of forelegs.

The controls all lived 17 days and were killed in formalin. They had an aver-

age length of 4.3 cm., maintained the tadpole body, and showed slight increase in the posterior leg buds as compared with the samples.

Thyroid 14.—All lived 17 days and were killed in formalin, had an average length of 4.2 cm., tadpole bodies, slightly better growth of the posterior leg buds than the controls, and no forelegs.

Thyroid 17.—All these were lost.

Thyroid 12.—One was lost. One died in 12 days, showing marked wasting and tail atrophy; considerable growth of the hind legs; left foreleg bud present; beginning frog-shaped body; length 2.5 cm. The third lived 17 days and was killed in formalin. This one was 3 cm. in length; the body was becoming frog-shaped; left foreleg bud present; tail atrophy about one-third.

Thyroid 15.—Two died in 11 days. Each measured 2.3 cm.; there was marked atrophy of the tail; posterior leg buds 5 mm. long; left foreleg bud present on one and not on the other; heads frog-shaped. The third died in 12 days; length 2.2 cm.; left foreleg present.

Thyroid 20.—All died in 10 days, measuring 2, 2.2, and 2.2 cm., respectively. All showed marked tail atrophy; left foreleg present on each; bodies becoming fairly frog-like.

This series shows that there is no qualitative difference in the action of desiccated thyroid on the different varieties of tadpoles used.

Series D.—May 27 to June 7, 1915 (duration, 12 days). (Fig. 4.)

For this experiment two sets of tadpoles of different ages with three in each set were used. The individuals of each age were alike as to size and condition of development. The older ones averaged 8.5 cm. in length, had posterior leg buds slightly over 2 cm. long, and had no forelegs. The younger set averaged 6.5 cm. in length, had just visible posterior leg buds and no foreleg buds. One of each age was killed in formalin at the beginning of the experiment for comparison. Another of each age was used for control and fed fresh liver every 2nd day. A third one of each age was fed 50 mg. of Thyroid 20 every 2nd day, alternating with fresh liver.

Both forelegs were present on the older thyroid-fed tadpole on the 7th day; the left foreleg appeared on the corresponding control on the 9th day. There was marked atrophy of the tail of the thyroid-fed tadpoles. The younger control had no forelegs; and the posterior leg buds were 8 mm. long; it maintained a tadpole body. The younger thyroid-fed animal had a frog-shaped head and body; left foreleg present; posterior legs 15 mm. long. The older tadpoles at the end of the experiment showed about the same amount of development of fore- and hind legs on both the controls and the thyroid-fed animal. There was marked tail atrophy in the latter and practically none in the former. The older thyroid-fed tadpole had become a well developed frog, while the corresponding control had the same characteristics to a slighter degree.

Comparison of samples, controls, and Thyroid 20 in Series C and D shows that desiccated thyroid of high iodine content administered to tadpoles of the same variety at different ages and stages of development produces the same effect; namely, immediate cessation of growth, rapid metamorphosis as evidenced by atrophy of the tail, increased growth of the posterior legs, and the development of forelegs. The younger the tadpoles at the beginning of thyroid feeding, the smaller the metamorphosed frog, and *vice versa*.

DISCUSSION AND CONCLUSIONS.

It seems evident from the foregoing experiments that the so called tumors (adenomata) of the thyroid possess the property of taking up iodine and metabolizing it into the active combination in the same way that the non-tumorous thyroid tissue does, although not so readily nor to the same degree, and the action on tadpoles of feeding desiccated tumorous thyroid tissue does not differ qualitatively from feeding desiccated non-tumorous thyroid tissue. The action in either case depends upon the iodine (active iodine) content, and in the case of the adenomata bears no constant relation to the state of their growth or differentiation.

Examination of Tables II and III shows that in the main this is true. There are, however, certain discrepancies as to time of death, appearance of first forelegs, degree of emaciation, and rate of growth in certain dishes of the series, the action being not quite parallel to the iodine content. Some of these discrepancies may be explained in part by accidents of feeding, slight differences in size, age, and susceptibility of the different tadpoles receiving the same thyroid, and also by the variations in the amount of thyroid consumed by the different individuals in the same dish. Lenhart has shown that the action of the same thyroid varies with the quantity fed. Another important factor which has to be considered is the condition of the iodine itself. It was suspected at the time of these experiments that the iodine might be present in an active and an inactive form, but no satisfactory proof of this assumption, at the begin ing of these experiments, was at hand. Support of this point has been afforded by the work of Kendall on the isolation of the active principle of thyroid and the separation of the iodine into two fractions. Since the com-

pletion of our experiments Marine[4] has demonstrated by means of perfusion experiments *in vivo* and *in vitro* that iodine is rapidly taken up by the thyroid cells, and though the iodine increase in the perfused lobe may be 1,000 per cent in 2 hours as compared with the control lobe, yet the action on tadpoles is no greater. It then becomes an important question to determine the time required by the thyroid to take up inorganic iodine and manufacture it into the active thyroid principle.

It is known that iodine is rapidly taken up by the thyroid, and in man the iodine content of the thyroid is subject to greater variations than in animals on account of the prevalent therapeutic use of iodine and the iodides in goiter and other conditions; even the iodine used in preparing patients for operations would increase the iodine content of the thyroid in a short time, so that one might expect such variations in the action of a given thyroid preparation fed to tadpoles as appear in these experiments.

In this connection it is interesting to note (Table II) that Thyroid 20 with 4.31 mg. of iodine was only slightly more active than No. 5 with 1.31 mg. of iodine. Two possibilities have to be considered here. First, No. 20 may have active iodine slightly greater than 1.31 mg. and the balance present as inactive iodine. Second, No. 5 with 1.31 mg. of iodine might represent the maximum possible effect under the conditions of the experiment and a larger quantity of active thyroid iodine could produce no greater effect.

Of course with the lower iodine contents the variations in effects might well come within the limits of errors of observation. Also the percentage error would be greater in the iodine determinations, accidents of feeding, etc.

Our conclusions as to the effect of feeding desiccated thyroid to tadpoles agree in general with those of Lenhart. The action of the thyroid depends not upon a specific stimulus to differentiation but upon a stimulation of metabolism in general in proportion to the active iodine and the quantity consumed. High iodine contents produce

[4] Marine, D., Demonstration *in Vitro* of the Specific Affinity of Thyroid Cells for Iodin, *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 132. Marine, D., and Feiss, H. O., The Absorption of Potassium Iodid by Perfused Thyroid Glands and Some of the Factors Modifying It, *J. Pharm. and Exp. Therap.*, 1915, vii, 557.

rapid emaciation, at the same time resulting in differentiation even in tadpoles dying in 8 to 12 days. Low iodine contents result in differentiation at an earlier period than the controls. Tadpoles fed on thyroid with practically no iodine grow better than the controls, in this instance the thyroid acting simply as a food.

Finally, the interest that the results of these experiments may have in connection with the question of function in tumor tissue should be pointed out. To those who hold that tumor lacks the capacity for physiological function, the adenomata of the thyroid could not be consistently regarded as tumors. To those who hold physiological function as a possible property of tumor tissue, the adenomata might be regarded as tumors. Future studies might warrant a recognition of different grades or degrees of tumor. On this basis the fetal adenoma (very little differentiation) might represent a higher degree of tumor than the diffuse colloid or simple adenomatous thyroid in which the adenomatous nodules are present to a great extent throughout the whole gland and are well differentiated. It is certain that there are all grades and degrees of growth and differentiation in the life history of fetal adenomata of the thyroid, from the pure fetal, undifferentiated adenoma with little or no iodine to the simple or colloid adenoma, well differentiated and with varying amounts of iodine approaching that of normal thyroid.

EXPLANATION OF PLATES.

Plate 38.

Fig. 1. Series A. The condition of the tadpoles at the time of death or after they had been killed in formalin, at the end of 31 days.

Plate 39.

Fig. 2. Series B. The condition of tadpoles at the time of death or after they had been killed in formalin, at the end of 49 days.

Plate 40.

Fig. 3. Series C. The effect of desiccated thyroid on *R. catesbiana* tadpoles as compared with the larvæ of *R. pipiens* and *R. clamata*.

Fig. 4. Series D. The effect of desiccated thyroid on tadpoles of different ages.

[illegible]

SPONTANEOUS DIABETES IN A DOG.

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PLATE 41.

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Spontaneous diabetes occurring in the dog or other lower mammal is of interest on account of its rarity; and when associated with pancreatic lesions, it becomes a valuable link between the experimental diabetes of dogs and the spontaneous diabetes of man. While some pathologists believe that it occurs frequently in overfed, pet dogs, numerous published records of diabetes in animals are not readily found.

Fröhner (1) observed seven dogs in which persistent glycosuria and other clinical signs of diabetes were present, and quotes other cases observed by Schindelka, Eichhorn, Miller and Fettick (three each), and Gutzeit and Lienaux, Thiermesse, Schmidt, Wolff, Haltenhoff, Schulz and Strubing (one each). Eber (2) reported a series of twelve animals, and Lanfranchi (3) and Ferraro (4) each one; but all these occurred before Opie had demonstrated the now well known lesions of the islands of Langerhans. In four of Eber's five cases changes were noted in the pancreas; in one the organ was reduced by chronic inflammation to one-half its normal size; and in the other three, of normal size, numerous small yellowish abnormal specks were visible. It is noteworthy that the animals in all these cases were of advanced age and that the symptoms closely resembled those of human diabetes. Case reports of diabetes in other domestic animals are even rarer, though it has been found in the horse by Dieckerhoff (5), Preller (6), Kruger (7), Heiss (8), Rueff (8), and Perosino (9); in the cow by Girotti (10), Ingardi (10), and Darbas (9); and in the ape by LeBlanc (9). Preller's careful study of a case of spontaneous diabetes in a horse is the only instance in which associated pancreatic lesions have been investigated by modern methods of technique. At autopsy, an interacinous pancreatitis with extensive degeneration of the islands of Langerhans was found. The type of degeneration, however, is not stated and no study of the island cells with special granule stains was made. As to the rarity of diabetes in animals Fröhner states that the two cases first observed by him were the only ones in 40,000 examinations (incidence of 0.005 per cent).

From the clinical point of view it is noteworthy, in comparing the experimental diabetes of animals with that of man, that one of Fröhner's and one of Eber's dogs went into diabetic coma 5 and 3 days, respectively, before death. In two other animals of Eber's series, the diacetic acid test was positive on several occasions. As ketonuria has been observed by Allen in certain types of experimental diabetes in dogs, such cases form an additional link between the experimental and the spontaneous form of the disease.

Case Report.

A pet Airedale bitch, weight 19 kilos, age 9 years, after pregnancy and abortion in the spring of 1915, began to lose weight. The loss of weight was progressive, the animal was continually thirsty, ate ravenously of an unrestricted dietary, and, as in the case of Naunyn's depancreatized dogs, it was noticed that flies collected about her urine. Diabetes mellitus was suspected and it was found on collecting urine that a single drop completely reduced Fehling's solution (about 2 cc.).

Dr. A. E. Taylor kindly consented to study the animal's metabolism; but when she was placed in the strange conditions of a metabolism cage many difficulties arose which prevented detailed studies. It was found, however, that on a general diet the animal eliminated 60 gm. of sugar per day. When placed on 10 gm. of nitrogen daily (beef heart), the amount of sugar was reduced to 30 gm., a G : N ratio of 3. When fasting, the G : N ratio sank to 1.7, and the daily sugar output fell below 10 gm. At no time was there any ketonuria. It is possible that if the fast could have been continued longer the animal would have become sugar-free; but she was so obviously uncomfortable under laboratory conditions that the owner preferred to have her returned to her home where instructions that she was to be fed a carbohydrate-free diet were carefully carried out. Her clinical condition, however, steadily grew worse; sugar continued in the urine in large amounts, and after 3 months, cataracts developed in both eyes to the stage of total blindness. Abscesses and ulcers occurred on the legs and trunk, and she became greatly emaciated. A rapidly growing, freely movable, painless tumor appeared in the right side of the neck, and soon discharged watery and later bloody pus. She was chloroformed at the suggestion of her owner 6 months after the first symptoms had been noticed.

Autopsy.—Weight 10.9 kg. The pancreas was removed immediately after death and small sections (about 2 mm. thick) were placed at once in Zenker's, formalin, and sublimate-bichromate solutions. The pancreas was found to be large, rather soft, of normal color and shape, and without any signs of old or recent inflammation or hemorrhage in or about it. Weight 33.8 gm.; length 14.5 cm.; width at head 4 cm.; at middle 3 cm. The tissue cut easily, disclosing the normal lobular arrangement, without increase of fibrous tissue. Numerous minute, ocher-yellow points (1 mm. or less in diameter) were seen through the peritoneal covering, especially near the tail, but these were less noticeable on the cut surface. Otherwise the postmortem examination showed nothing worthy of note except a tumor, 83 gm. in weight, occupying the right side of the neck at the level of the thyroid cartilage but arising apparently in the thymus gland. A similar mass 2.5 cm. in diameter was found at the base of the heart.

Histology of the Pancreas.—The acini are well filled with zymogen, in some places so densely packed that these areas have an almost homogeneous reddish appearance (hematoxylin and eosin) with the acinous cells much obscured. In a few areas are collections of small round cells with localized increase of fibrous tissue, but these changes are not sufficiently numerous or extensive to be important. The blood vessels are normal. Striking abnormalities, however, are found in the islands of Langerhans. Not only are they scarce, but every island shows degeneration of one or more kinds (Fig. 1). The most obvious change, easily visible in almost every island, is the typical hydropic degeneration of one or more cells. Similar hydropic cells are prominent, either singly or in groups of three or four, in the interacinous spaces. These are probably miniature islands of Langerhans. As we did not have the necessary stain, the tissues fixed in sublimate-bichromate solution were sent to Mr. Walter B. Martin, of the Johns Hopkins Medical School, who kindly prepared sections stained with neutral azo-violet, to bring out the *alpha* and *beta* granule cells of the islands of Langerhans. Although the fixative did not penetrate for any great depth into the tissue, he was able to observe the changes in the islands fairly well, and reports as follows:

"The islets show complete degeneration of the *beta* cells, although remnants of these cells still are apparent in some of the islands as red staining areas. The *alpha* cells are also undergoing degeneration, but this has not progressed so far as in the others. The outline of the *alpha* cells can be made out, their nuclei are intact, and in a number of cases, the blue granulation is still quite distinct. Some of the islets are made up entirely of *alpha* cells undergoing degeneration."

Although some of the island cells have a hyaline appearance, the typical hyaline degeneration of Opie (11) and Weichselbaum (12) is not present. On the other hand, certain localized areas of fibrous tissue, especially those containing one or two isolated hydropic cells, show that in many cases whole islands have been replaced by fibrous tissue.

Histologic examination of the growth in the neck shows it to be a sarcomatous tumor of the thymus. The second tumor is a metastasis of the same in a lymph node. Thyroid, pituitary, adrenals, spleen, and liver show nothing of importance histologically, except extreme fatty change in the last named organ.

SUMMARY.

The picture is one of a true diabetes mellitus, first attracting attention after miscarriage. Although the G : N ratio sank from 3 : 1 on 10 gm. of nitrogen to 1.7 : 1 after 3 days' fasting, the disease progressed steadily in spite of a long continued carbohydrate-free diet. Together with the usual complications of diabetes, a malignant tumor of the thymus developed, so that after 5 months' observations a slow death was forestalled by chloroform. The most striking feature at autopsy was the large, apparently normal pancreas, which exhibited histologically marked changes in the islands of Langerhans, extreme hydropic degeneration and exhaustion of granules, involving both *alpha* and *beta* cells, but especially the latter, and replacement of some islands by fibrous tissue.

BIBLIOGRAPHY.

1. Fröhner, E., in Friedberger, F., and Fröhner, E., Veterinary Pathology, London, 6th edition, 1908, ii, 133; *Monatsh. prakt. Thierheilk.*, 1891–92, iii, 149.
2. Eber, W., *Monatsh. prakt. Thierheilk.*, 1897–98, ix, 97.
3. Lanfranchi, quoted by Fröhner, E., and Zwick, W., Lehrbuch der speciellen Pathologie und Therapie der Hausthiere, Stuttgart, 8th edition, 1915, i, 820.

4. Ferraro, P., *Morgagni*, 1885, xxvii, 103.
5. Dieckerhoff, W., *Berl. thierärztl. Woch.*, 1892, viii, 457.
6. Preller, Dissertation, Bern, 1908.
7. Kruger, quoted by Preller, Dissertation, Bern, 1908.
8. Heiss and Rueff, quoted by Dieckerhoff, *Berl. thierärztl. Woch.*, 1892, viii, 457.
9. Perosino, Darbas, and LeBlanc, quoted by Fröhner in Friedberger and Fröhner, Veterinary Pathology, London, 6th edition, 1908, ii, 133.
10. Girotti and Ingardi, quoted by Fröhner and Zwick, Lehrbuch der speciellen Pathologie und Therapie der Hausthiere, Stuttgart, 8th edition, 1915, i, 820.
11. Opie, E. L., *J. Exp. Med.*, 1900–01, v, 398, 527.
12. Weichselbaum, A., *Wien. klin. Woch.*, 1911, xxiv, 153.

EXPLANATION OF PLATE 41.

FIG. 1. Degenerated island of Langerhans. *a*, two cells showing extreme hydropic degeneration; *b*, normal island cells with *alpha* granules; *c*, two cells with *beta* granules that have escaped destruction. (In very few islands of this pancreas are these cells to be found.) *d*, island cells showing beginning hydropic degeneration; *e*, pancreatic acinus with lumen and apices of acinus cells filled with zymogen; *f*, fibrosis, involving most of the island; *g*, pancreatic acinus in which zymogen granules have failed to take the stain.

Fixation, sublimate-bichromate. Stain, Martin's azo-fuchsin-neutral violet.

366

TECHNIQUE OF CULTIVATING HUMAN TISSUES IN VITRO.

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In 1910 Carrel and Burrows[1] reported the successful cultivation *in vitro* of a human sarcoma. They used as a culture medium plasma from the patient from whom the tumor was removed, and observed an active migration of cells during several days' incubation. No subcultures were made. In subsequent attempts to cultivate human tissue Carrel[2] experienced considerable difficulty owing apparently to the liquefaction of the clotted plasma medium. He noted, as did also Lambert and Hanes,[3] Maccabruni,[4] and others, that within 24 hours a clear liquefaction zone appears around each tissue fragment, and that after a few days the entire fibrin clot becomes liquefied. Unless the cells wander out early they find no framework upon which to grow.

Losee and Ebeling[5, 6] attempted various modifications of human plasma with the object of preventing liquefaction, but were unsuccessful. However, by diluting the plasma with Ringer's solution, which seemed to delay digestion, and by making transfers of the tissue fragment to fresh plasma every 24 to 48 hours, they were able in a few instances to propagate human connective tissue cells obtained from fetuses through a number of subcultures, in one case as long as 60 days. They attributed their success in part to the addition of tissue extracts to the medium, as recommended by Carrel.[7] They emphasized the shortcomings of

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[1] Carrel, A., and Burrows, M. T., Human Sarcoma Cultivated outside of the Body, *J. Am. Med. Assn.*, 1910, lv, 1732.

[2] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[3] Lambert, R. A., and Hanes, F. M., The Cultivation of Tissue in Plasma from Alien Species, *J. Exp. Med.*, 1911, xiv, 129.

[4] Maccabruni, F., Esperienzi di coltivazioni "in vitro" del cancro uterino umano, *Ann. ostet. e ginecol.*, 1914, xxxvi, pt. i, 57.

[5] Losee, J. R., and Ebeling, A. H., The Cultivation of Human Tissue *in Vitro*, *J. Exp. Med.*, 1914, xix, 593.

[6] Losee and Ebeling, The Cultivation of Human Sarcomatous Tissue *in Vitro*, *J. Exp. Med.*, 1914, xx, 140.

[7] Carrel, Artificial Activation of the Growth *in Vitro* of Connective Tissue, *J. Exp. Med.*, 1913, xvii, 14.

their technique and stated that they experienced many failures, for which the early rapid liquefaction of the fibrin was held responsible.

In our first efforts to cultivate human tissues in which unmodified human plasma was used as a culture medium, the results were practically the same as those of Carrel, and Losee and Ebeling. In only exceptional cases were we able to obtain satisfactory growths. There was regularly rapid digestion of the fibrin about the pieces of tissue, thus completely blocking cell migration. We therefore undertook to overcome this difficulty. It may be noted here that it is not the ability of human tissue to digest fibrin, but rather the susceptibility of human fibrin to digestion which is the basis of the trouble, for we found that human tissue did not liquefy the fibrin of foreign plasmas, while it was observed that human fibrin was readily digested by the ferments of practically every foreign tissue.

In the cultivation of the tissues of a number of lower animals,—rats, mice, guinea pigs, rabbits, dogs, cats, fowls, and pigeons,—using the plasma from these and other species, liquefaction was not infrequently observed, though never so regularly or extensively as in cultures containing human plasma. It was noted, moreover, that fowl and pigeon plasma were never liquefied, except in the presence of particular types of bacteria. Since earlier experiments had shown that tissues from certain of the lower animals could be cultivated in plasma from foreign species,[3] it occurred to us that this property of fowl and pigeon plasma to resist digestion might be utilized in growing human tissues. It seemed possible that chick fibrin might be made to serve as the framework of the culture medium with human serum or plasma added to supply the necessary nutritive substances. A medium was therefore prepared by mixing a small quantity of chick plasma with a considerably larger quantity of human serum. Various human tissues (lymph gland, spleen, skin, etc.) obtained at operation were put up in this mixed medium. No liquefaction was observed, and active progressive growth with numerous mitotic figures was seen in the majority of the preparations where soft friable tissue was used. Tissues removed at autopsy several hours after death were cultivated, the preparations showing in some instances a very active growth of connective tissue cells. It was not necessary

to make transfers to fresh plasma oftener than every 5 days. Continued propagation through subcultures was carried on for several months with as little difficulty as in the cultivation of similar tissues from lower animals. It may be noted that human tissues will grow in pure fowl plasma, but growth is not so active or so prolonged as in the modified medium containing human serum. Various proportions of fowl plasma and human serum were tested with the object of working out an optimum medium. A mixture of chick plasma, one part, and human serum, four parts, seemed to yield the best growth. The use of a relatively small amount of plasma has also an economic advantage in that human serum may obviously be obtained more easily and in larger quantity than fowl plasma. Furthermore, serum is easily preserved, whereas plasma sometimes clots after a few hours, even when kept cold in paraffined receptacles. It may be suggested that a still smaller proportion of plasma can be used since one part of plasma to twenty of serum will form a fairly firm clot.

The human serum used in these preliminary experiments came from a single individual. Autogenous plasma was not used since studies upon the cultivation of the tissues of lower animals had shown that plasma from the animal supplying the tissue was no better as a culture medium than plasma obtained from other members of the same species. For example, it has been found that the cells of transplantable mouse and rat tumors would grow quite as well in the plasma of rats and mice artificially or naturally immune to these tumors as in the plasma of tumor-bearing animals.[8] It seemed conceivable, however, that human tissues might prove more sensitive to individual variations in the culture medium. While studying this question it occurred to us that the influence of naturally existing iso-antibodies (agglutinins and hemolysins) upon growth *in vitro* might be investigated at the same time.

The interagglutinating reactions of human bloods, due, as Landsteiner and Leiner[9] and others have shown, to the existence of two

[8] Lambert and Hanes, A Study of Cancer Immunity by the Method of Cultivating Tissues outside the Body, *J. Exp. Med.*, 1911, xiii, 505.

[9] Landsteiner, K., and Leiner, K., Ueber Isolysine und Isoagglutinine im menschlichen Blut, *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxviii, 548.

agglutinins and two agglutinogens in four different combinations[10] are well known. By simple agglutination tests any individual is found to fall into one of four groups depending on the power of his serum to agglutinate the blood of certain other persons, and on the susceptibility of his blood to agglutination; that is, on the presence or absence of one or both of the two agglutinins and agglutinogens.

For our experiments serum was obtained from individuals belonging to each of the four groups, and from the patients whose tissues were used for cultivation. The following protocol is illustrative of the results obtained.

Experiment 210.—An axillary lymph gland, removed at operation and showing microscopically changes characteristic of Hodgkin's disease, was placed in a sterile dish of cold salt solution. 1 hour later tissue cultures were put up in each of the following sera, to which chick plasma was added in the proportion of one part of plasma to four of serum: Group I, Group II, Group III, Group IV, and patient's serum (Group II). Ten cultures of each series were prepared and examined daily during the following week of incubation. Within 24 hours active migration of large mononuclear wandering cells and leukocytes was noted in practically every culture. On the 2nd and 3rd days a beginning outgrowth of connective tissue cells was observed, reaching a maximum activity about the 5th day. At no time could any difference in the five series be detected. The usual variations in individual cultures were seen, but the average extent of outgrowth was practically the same for each series. There was likewise no difference in the frequency of mitotic figures, which can be readily recognized by the practised observer in the living unstained preparations.

Other experiments in which tissues from Group I and Group III individuals were used gave the same result. While we were unable to secure any tissue from a person belonging to Group IV, as such

[10] It is assumed that there exist in human sera two agglutinins, *a* and *b*, and two corresponding agglutinogens, *A* and *B*. The blood of Group I possesses both agglutinins but no agglutinogens. It therefore agglutinates the blood of all other groups, but is not agglutinated by any serum. Group II possesses agglutinin *a* and agglutinogen *B*. It agglutinates blood of Group III and Group IV, and is agglutinated by members of Group I and Group III. Group III, the reciprocal of Group II, possessing agglutinin *b* and agglutinogen *A*, is agglutinated by Group I and Group II sera, and agglutinates the corpuscles of the third and fourth groups. Group IV contains no agglutinin but has both agglutinogens. Its serum therefore has no agglutinating power, but its corpuscles are agglutinated by the sera of all other groups.

people are rare, we feel justified in concluding that normally existing iso-antibodies in human blood do not influence in any way the growth of human tissues *in vitro*.

In addition to the question of culture medium we have been concerned with another problem in connection with the technique of cultivating human tissues *in vitro;* namely, the preservation of tissues after removal from the body. It is often not convenient to prepare cultures immediately upon the receipt of the tissue from the operating room or autopsy table. It is also desirable in many cases to carry out a number of experiments with the same tissue, not possible in a few hours' time. Furthermore, the duration of life of tissues in cold storage is a question of biological interest as well as of practical importance. In order to determine the period of survival under conditions of ordinary ice box preservation, cultures of the tissue were made on the day of removal, and at 2 day intervals thereafter, up to 12 days. A type experiment is described in the following protocol.

Experiment 320.—A piece of small tumor removed surgically from the region of the parotid gland (microscopically, a typical mixed tumor) was placed immediately in the ice box in a dish of physiological salt solution (0.8 per cent). Half an hour later the tumor, which was composed of soft friable tissue, rather gelatinous in places, was cut into small pieces. From some of these, cultures were made immediately. The remainder, covered with salt solution in a Petri dish, were returned to the ice box, an ordinary, small wooden structure with a lid, the temperature of which fluctuated between 10° and 15°C. The ten cultures put up on the day of operation grew well, showing after 2 to 3 days' incubation numerous large cells of irregular shape with frequent mitotic figures. Cultures made on the 2nd and 4th days of preservation, showed similar activity, though in the case of the tissue preserved for the longer time there was a longer latent period before migration was observed. Tissue kept for 6 days showed growth in the majority of preparations, though not so good as those put up on the 4th day. In the 8 day series only a few active cells were seen. Preparations of tissue kept for 10 and 12 days were all negative.

In another experiment in which a piece of lymph gland was used, no cells survived longer than 6 days. A glioma showed some growth after 8 days' preservation, but none after 10 days.

These experiments show that under very simple conditions of preservation human connective tissue cells and the cells of certain benign

tumors remain alive in the ice box for 6 to 8 days at least. 10 days probably represents the limit of survival. The period of survival is approximately the same as that determined for normal rat connective tissue, though somewhat shorter than that for embryonic chick tissues, which previous experiments have shown live 8 to 16 days in storage.[11] The optimum temperature for chick tissues was found to be about 6°C. The optimum temperature for preserving human tissues was not determined.

CONCLUSIONS.

1. Unmodified human plasma is not a satisfactory culture medium for human tissues owing to the susceptibility of human fibrin to digestion by tissue ferments. The necessary framework is thus destroyed before the cells begin to migrate. The difficulty can be overcome by adding to human plasma or serum a small quantity of fowl or pigeon plasma, the fibrin of which is highly resistant to digestion. Human tissues have been propagated in this medium for several months through subcultures, and growth *in vitro* can probably be maintained indefinitely.

2. Human tissues show no greater sensitiveness to changes in temperature and mechanical injury associated with preparation of cultures than those of lower animals. They may be preserved in an ordinary ice box at 10–15°C. as long as 6 or 8 days. Tissues obtained at operation give best results, but pieces of organs removed at autopsy 1 to 4 hours after death sometimes show active growth.

3. The presence of normally existing iso-antibodies (agglutinins and hemolysins) in human serum is without influence on the growth of human tissues *in vitro*. In other words, autogenous serum has no advantage in tissue cultures over homologous serum.

For the agglutination tests carried out in connection with the experiments reported, I am indebted to Miss M. P. Olmstead of the bacteriological laboratory of the Presbyterian Hospital.

[11] Lambert, R. A., The Influence of Temperature and Fluid Medium on the Survival of Embryonic Tissues *in Vitro*, *J. Exp. Med.*, 1913, xviii, 406.

A STUDY OF SERUM SALVARSANIZED IN VITRO.

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INTRODUCTION.

In a previous communication, Ellis and I[1] reported that the serum of patients who were treated intravenously with salvarsan or neosalvarsan had a definite spirocheticidal action. This action was increased by heating the serum at 56°C. for one-half hour. By an indirect method it was shown, first, that the heat destroyed some inhibitory substance in the serum, and, second, that it directly increased the action of the serum-salvarsan mixture. The beneficial effects of intraspinal injections of serum in the treatment of syphilis of the central nervous system have been attributed in part to this spirocheticidal substance. Because of the variable quantities of salvarsan in the serum of patients after intravenous injections of the drug, Ogilvie[2] has suggested a method for the preparation of salvarsanized serum in which a small quantity of faintly alkaline solution of salvarsan is added to serum, the mixture incubated and heated at 56°C. for one-half hour. The object of the present work was to study the spirocheticidal effect of serum salvarsanized *in vitro*.

Following our first communication, Stühmer[3] showed that the serum of salvarsan-treated rabbits had a trypanosomicidal action which was markedly increased by heating. He also showed that heating increased the intensity of the paradimethylamidobenzaldehyde color reaction for salvarsan, and that the intensity of the color reaction and the trypanosomicidal power were more or less parallel. However, by studying the serum daily for 10 days following the intravenous injection of salvarsan, he demonstrated that the trypanosomicidal action of heated serum persisted several days after the color reaction had disappeared. He[4] next showed that the trypanosomicidal action of the serum of salvarsan-treated rabbits persisted longer than that of neosalvarsan-treated rabbits, and that the serum of rabbits treated intramuscularly with salvarsan had very little action. Ehrlich

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[1] Swift, H. F., and Ellis, A. W. M., *J. Exp. Med.*, 1913, xviii, 435.

[2] Ogilvie, H. S., *J. Am. Med. Assn.*, 1914, lxiii, 1936.

[3] Stühmer, A., *Münch. med. Woch.*, 1914, lxi, 745.

[4] Stühmer, *Münch. med. Woch.*, 1914, lxi, 1101.

suggested that the effect of heat was to break up a loose combination which had taken place between the salvarsan and some substance in the serum, thus setting free the salvarsan which acts on the trypanosomes or spirochetes; but Stühmer[5] attributed the increased action of heated serum to an increased oxidation. He finally studied the effect of oxidation and reduction on the salvarsanized rabbit serum and showed that oxidation did not increase the trypanosomicidal power of either unheated or heated serum, and also that reduction with weak sodium hydrosulphite solution did not decrease the power of the unheated serum. Reduction, however, did affect the heated serum by removing the increased trypanosomicidal action which had been produced by heating. In other words, the reduction caused the heated serum to act in the same manner as unheated serum. As Stühmer's experiments were not performed with diminishing dilutions of serum, but with serum obtained on successive days after the administration of salvarsan, the exact relation of heating, oxidation, and reduction are not entirely clear.

Methods.

In the present study a strain of *Spirochœta duttoni*, propagated in white mice, has been employed. The suspension for performing the test was prepared by bleeding a well infected mouse into warm normal saline solution, and diluting with normal saline solution until the required number of spirochetes per field was obtained. Equal quantities of this suspension and the dilution of serum to be tested were mixed thoroughly and incubated at 37°C. for 1 hour. 1 cc. of the mixture, representing 0.5 cc. of the spirochete suspension, was injected into mice intraperitoneally. The blood of the infected mice was examined daily with the dark-field microscope. The intensity of the subsequent infection is indicated by the average count of twenty fields, as shown in the protocols. The salvarsanized serum was prepared as follows: A slightly alkaline watery solution of salvarsan was diluted to 1:2,000 with 0.5 per cent saline solution. One part of this was added to nine parts of serum, making a dilution of 1:20,000, and from this the subsequent dilutions were made with normal serum, or normal salt solution, as the experimental conditions required.

EXPERIMENTAL.

The serum-salvarsan mixture prepared *in vitro* had a definite spirocheticidal action. This point was previously demonstrated by

[5] Stühmer, *Münch. med. Woch.*, 1914, lxi, 2338.

TABLE I.

Comparison of Serum Salvarsanized in Vitro with Serum of a Salvarsan-Treated Patient.

Mouse.	Dilution.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.				
				1	2	3	4	5
	Salvarsan.	*mg.*						
A1	1:40,000	0.025	Unheated.	0	0	±w	0	0
A2	1:80,000	0.012	"	±w	+	+++	0	0
A3	1:160,000	0.006	"	±	+	+++	0	0
A4	1:320,000	0.003	"	±	++	+++	0	0
A5	1:640,000	0.0015	"	±w	++	+++	0	0
B1	1:40,000	0.025	Heated.	0	0	0	0	0
B2	1:80,000	0.012	"	0	0	0	0	0
B3	1:160,000	0.006	"	±w	+	+	0	0
B4	1:320,000	0.003	"	±	+	+++	0	0
B5	1:640,000	0.0015	"	+	+	+++	0	0
	Patient's serum.							
C1	1:1		Unheated.	±w	±	±w	0	0
D1	1:1		Heated.	±w	±	0	0	0
D2	1:2		"	±	++	±	0	0
E1	1:1		Unheated.	±	++	+	0	0
F1	1:1		Heated.	±w	++	±	0	0
F2	1:2		"	±	++	+	0	0
Control. S1	1:1			±w	++	+++	0	0

±w indicates between 1 and 19 spirochetes in 20 fields; ±, 1 per field; +, 2–10 per field; ++, 11–20 per field; +++, 20–50 per field; ++++, more than 50 per field.

Dilutions of salvarsan in normal serum were made, incubated, and each dilution divided into two portions; one of each portion was heated at 56°C. for one-half hour. A syphilitic patient weighing 71 kilos received 0.3 gm. of salvarsan intravenously (Sera C and D), and another weighing 68 kilos received 0.5 gm. (Sera E and F). Blood was taken from each after 1 hour, allowed to coagulate, centrifugalized, and the serum removed immediately. The serum from each was divided into two portions, one of which was heated at 56°C. for one-half hour, and then diluted as indicated. The spirochetal suspension, containing between six and eight spirochetes per field was mixed with the various sera; it was then incubated for 1 hour and injected into mice.

Gonder.[6] Heating the mixture at 56°C. for one-half hour increased this action. The effect of heating was studied in a series of seven experiments; four times the heating doubled the strength of the serum, twice it quadrupled it, and once increased it eightfold. In terms of absolute amounts of salvarsan in the heated serum, in three instances the spirocheticidal action was complete in a concentration of 0.025 mg., in three others of 0.012 mg., and once of 0.006 mg. per cc. of serum. This variation was probably due to the difference in the number of spirochetes in the suspension in the several experiments. In Experiment 1 (Table I) the heated serum salvarsanized *in vitro* was more effective in a dilution of 1:80,000 than the patient's serum, and the heating had less relative influence on the patient's serum. It should be noted here that the serum was removed from the clot within an hour of the time the patient was bled. In this respect this part of the experiment differs from those previously reported. In our earlier work the serum always stood over night before separation from the coagulum.

Although the spirocheticidal action of the unheated serum which was separated immediately was only slightly less than that which stood on the clot over night, a greater difference was noted between the two sera after they were heated. Heating increased the action of the serum which had been in contact with the clot over night much more than it did the serum removed from the clot immediately (Table II). Six different experiments were performed with the serum of patients obtained after salvarsan treatment. In four in which the serum was separated immediately, the increase in spirocheticidal action of heated serum was considerably less marked than in two in which the serum was removed from the clot 24 hours after the bleeding (Table VII, G and H).

After the demonstration of this point with the serum of salvarsan-treated patients, it seemed desirable to determine whether the serum from blood salvarsanized *in vitro* would act in the same manner. The results of this experiment are shown in Table III. Here the two unheated sera were again spirocheticidal in the same dilution, although Serum A in 1:2 dilution inhibited the infection 2 days

[6]Gonder, R., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 309.

TABLE II.

Comparative Effect of Immediate Separation from Clot and Separation after Standing 24 Hours, with Serum from Salvarsan-Treated Patient.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.			
			1	2	3	4
	Separated immediately.					
A1	1:1	Unheated.	+	+++	Dead.	—
A2	1:2	"	+	+++	±w	0
B1	1:1	Heated.	0	±	++	+++
B2	1:2	"	+	+	±	0
B3	1:4	"	±w	+	+	0
	Separated after 24 hrs.					
C1	1:1	Unheated.	+	++	+++	0
C2	1:2	"	+	+++	+	0
D1	1:1	Heated.	0	±w	+	±w
D2	1:2	"	0	±w	+	0
D3	1:4	"	±w	±w	+	±
Control.						
S1	Injected immediately.		+	++++	0	0

A patient weighing 65 kilos received 0.4 gm. of salvarsan intravenously. 50 cc. of blood were withdrawn after one-half hour, allowed to coagulate, centrifugalized, and 10 cc. of serum pipetted off. The remainder of the serum was allowed to stand on the clot over night, after which it was centrifugalized and pipetted off. Each serum was divided into two lots, and one of each heated at 56°C. for one-half hour. Dilutions of each series were made with normal saline; the spirochetal suspension containing from four to five spirochetes per field was added; it was then incubated for 1 hour and injected into mice.

longer than did Serum C in the same solution. When the two sera were heated, however, the one that was immediately removed from the clot was more potent than the one that stood on the clot over night. This is the opposite of what occurred with patient's serum. The explanation of this difference is not clear, but it is suggested that the salvarsan undergoes some change while circulating in the body which it does not undergo when it is mixed with blood in the test-tube. This may be due to the fact that in the body it is in contact with blood in the fluid condition for a longer period, hence oxidative conditions are more complete; or that contact with the tissues brings

TABLE III.

Comparative Effect of Immediate Separation from Clot and Separation after Standing 24 Hours with Serum from Blood Salvarsanized in Vitro.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.						
			1	2	3	4	5	6	7
	Serum separated immediately.								
A1	1:1	Unheated.	0	Dead.*	–	–	–	–	–
A2	1:2	"	0	0	0	0	±	±w	±
A3	1:4	"	0	±	++	++	Lost.	–	–
A4	1:8	"	±w	±	++	0	0	0	0
B2	1:2	Heated.	0	0	0	0	0	0	0
B3	1:4	"	0	0	0	0	0	0	0
B4	1:8	"	0	±w	+	+++	0	0	0
B5	1:16	"	±w	+	++	+	0	0	0
	Serum separated after standing over night.								
C1	1:1	Unheated.	0	0	0	0	0	0	0
C2	1:2	"	0	0	±w	+	0	0	0
C3	1:4	"	0	±w	±w	0	0	0	0
C4	1:8	"	0	+	++	0	0	0	0
D2	1:2	Heated.	0	0	0	0	0	0	0
D3	1:4	"	0	0	±w	±	±w	0	0
D4	1:8	"	0	±w	+	±w	0	0	0
D5	1:16	"	±w	++	+++	+++	0	0	0

* Died from mouse typhoid infection.

2 cc. of a 1:2,000 dilution of salvarsan were mixed with 18 cc. of freshly drawn human blood. This was divided into two equal portions and allowed to coagulate. The first was then centrifugalized and the serum removed from the clot; the second was allowed to stand over night in the ice box, after which it was centrifugalized and the serum removed. Both lots of serum were divided into two portions, and one of each was heated at 56°C. for one-half hour. Dilutions of each of the four series were made with normal saline solution. The suspension of spirochetes, containing three to four spirochetes per field, was added; it was then incubated for 1 hour and injected into mice.

about some change in it which is not effected in the test-tube. From this point of view, at least, the use of serum of salvarsan-treated patients seems justified.

TABLE IV.

Comparison of Serum Salvarsanized in Vitro with Serum from Blood Salvarsanized in Vitro.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.			
			1	2	3	4
	Salvarsanized serum.					
A1	1:1	Unheated.	0	0	0	0
A2	1:2	"	±w	±	+	0
A3	1:4	"	±	++	+	0
A4	1:8	"	+	++	+	Dead.
B1	1:1	Heated.	0	0	0	0
B2	1:2	"	0	0	0	0
B3	1:4	"	0	0	0	0
B4	1:8	"	±w	+	±	0
B5	1:16	"	±w	+	+	±w
	Serum from salvarsanized blood.					
C1	1:1	Unheated.	0	±w	±w	+
C2	1:2	"	0	±w	+	±w
C3	1:4	"	±w	++	+	0
C4	1:8	"	+	++	+	0
D1	1:1	Heated.	0	0	0	0
D2	1:2	"	0	0	0	0
D3	1:4	"	±w	±	++	0
D4	1:8	"	±w	+	++	0
D5	1:16	"	+	++	+++	+
Controls.						
S1	Injected immediately.		±w	++	Dead.	
S2	After 1 hr. incubation with normal sera.		+	++	"	

1 cc. of a 1:2,000 dilution of salvarsan was mixed with 9 cc. of normal human serum (making a dilution of 1:20,000), incubated for 1 hour, divided into two portions, A, unheated, and B, heated, at 56°C. for one-half hour. 1 cc. of the same salvarsan dilution was mixed with 9 cc. of freshly drawn blood, incubated for 1 hour, centrifugalized, and the serum pipetted off and divided into two portions, C, unheated, and D, heated the same as B. The various series were then diluted with normal saline solution; the spirochetal suspension, containing eight to ten spirochetes per field, was added; it was then incubated for 1 hour and injected into mice.

Experiment 4 (Table IV) was devised to determine whether a more potent serum resulted from the addition of salvarsan to serum or to blood. Both were prepared on the same day and tested within 2 hours of the time of preparation. In both the unheated and heated condition the serum prepared by the direct addition of salvarsan was more spirocheticidal than the serum from blood to which salvarsan had been added.

The demonstration that heating increased the spirocheticidal action of the salvarsan-serum mixture permitted a study of the effect of heat on each component of the mixture. This experiment confirmed by a direct method what was previously demonstrated by an indirect method, that part of the action of the heat was to remove some inhibitory substance from unheated serum. In Series C, Table V, the heating of the serum before the salvarsan was added resulted in producing a more active mixture than in Series A where the mixture was not subjected to heat. But the action of the mixture in Series C was not so strong as in Series B where the two were heated together. Series D demonstrated that part of the effect of the heat was directly upon the salvarsan, for although the heated salvarsan was combined with unheated serum, which contained inhibitory substances, the mixture was more spirocheticidal than the unheated mixture, but again not so active as the heated mixture. That the total result of the heat is due to a summation of these two effects is shown in Series E where the two components were combined after heating. This mixture was stronger than either C or D and had practically the same effect as in Series B where the two were heated together; the only exception noted was in Mouse E4 where there was a very slight infection on the 3rd day.

The relative effect of heat upon removal of inhibitory substance in the serum and upon increasing the spirocheticidal action of salvarsan was only slightly brought out in this experiment, where the infection of Mouse C4 was less than that of D4; but in two other similar experiments, in which the controls showed a more intense infection, the 1:80,000 mouse in the C series was completely protected, while the same mouse in the D series showed distinct infection. The effect of the heat seems, therefore, to be relatively greater in the removal of inhibitory substance from the serum than in directly increasing the activity of the salvarsan.

TABLE V.

Effect of Heat on Each Component of the Serum-Salvarsan Mixture.

Mouse.	Dilution of salvarsan.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.				
				1	2	3	4	5
		mg.						
A1	1 : 20,000	0.05	Unheated.	0	0	0	0	0
A2	1 : 40,000	0.025	"	0	0	0	± w	±
A3	1 : 80,000	0.012	"	± w	+	+	0	0
A4	1 : 160,000	0.006	"	± w	+	± w	0	0
A5	1 : 320,000	0.003	"	+	+++	+	0	0
B1	1 : 20,000	0.05	Serum and salvarsan heated after mixing.	0	0	0	0	0
B2	1 : 40,000	0.025		0	0	0	0	0
B3	1 : 80,000	0.012		0	0	0	0	0
B4	1 : 160,000	0.006		0	0	0	0	0
B5	1 : 320,000	0.003		±	+	++	0	0
C1	1 : 20,000	0.05	Serum heated, salvarsan unheated.	0	0	0	0	0
C2	1 : 40,000	0.025		0	0	0	0	0
C3	1 : 80,000	0.012		0	0	0	0	0
C4	1 : 160,000	0.006		± w	± w	± w	0	0
C5	1 : 320,000	0.003		±	+	+++	± w	0
D1	1 : 20,000	0.05	Serum unheated, salvarsan heated.	0	0	0	0	0
D2	1 : 40,000	0.025		0	0	0	0	0
D3	1 : 80,000	0.012		0	0	0	0	0
D4	1 : 160,000	0.006		± w	±	+	0	0
D5	1 : 320,000	0.003		± w	+	0	0	0
E1	1 : 20,000	0.05	Serum and salvarsan heated separately before mixing.	0	0	0	0	0
E2	1 : 40,000	0.025		0	0	0	0	0
E3	1 : 80,000	0.012		0	0	0	0	0
E4	1 : 160,000	0.006		0	0	± w	0	0
E5	1 : 320,000	0.003		+	+	0	0	0
Controls.								
S1	Injected immediately.			+	++	+	0	0
S2	Incubated with normal serum.			+	++	0	0	0

The serum was from a patient with cured (?) syphilis. Wassermann reaction negative. Series A and B were prepared as in Table I. In Series C the serum was heated at 56°C. for one-half hour and then the salvarsan was added and dilutions were prepared. In Series D the salvarsan (1 : 2,000 dilution) was heated at 56°C. and then diluted with unheated serum. At the end of the period of heating the salvarsan was only very slightly turbid. Portions of the heated serum of Series C and heated salvarsan of Series D after heating were mixed in the usual way. The spirochetal suspension, containing between four and six spirochetes per field, was mixed with the various sera; it was then incubated for 1 hour and injected into mice.

TABLE VI.

Comparison of Salvarsanized Serum with Neosalvarsanized Serum.

Mouse.	Dilution.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.			
				1	2	3	4
		mg.					
	Salvarsan.						
A1	1 : 20,000	0.05	Unheated.	0	0	0	0
A2	1 : 40,000	0.025	"	0	0	0	0
A3	1 : 80,000	0.012	"	± w	±	+	0
A4	1 : 160,000	0.006	"	± w	+	+	0
B1	1 : 20,000	0.05	Heated.	0	0	0	0
B2	1 : 40,000	0.025	"	0	0	0	0
B3	1 : 80,000	0.012	"	0	0	Dead.*	—
B4	1 : 160,000	0.006	"	0	± w	+	0
B5	1 : 320,000	0.003	"	± w	+	++	0
	Neosalvarsan.						
C1	1 : 20,000	0.05	Unheated.	0	0	0	0
C2	1 : 40,000	0.025	"	0	0	0	0
C3	1 : 80,000	0.012	"	0	0	0	0
C4	1 : 160,000	0.006	"	± w	± w	+	0
C5	1 : 320,000	0.003	"	±	++	+++	+
D1	1 : 20,000	0.05	Heated.	0	0	0	0
D2	1 : 40,000	0.025	"	0	0	0	0
D3	1 : 80,000	0.012	"	0	0	0	0
D4	1 : 160,000	0.006	"	0	0	0	0
D5	1 : 320,000	0.003	"	0	± w	++	0
Control.							
S1	Injected immediately.			+	++++	+	0
S2	After 1 hr. incubation with normal serum.			+	++++	0	0

* Died from mouse typhoid infection.

Dilutions of salvarsan were made in normal serum in the usual way. Similar dilutions of neosalvarsan in serum were prepared, 0.15 gm. of neosalvarsan being considered the equivalent of 0.1 gm. of salvarsan. Each dilution was divided into two portions, one of which was heated at 56°C. for one-half hour. The spirocheticidal suspension, containing six to eight spirochetes per field, was mixed with the sera; it was then incubated and injected into mice.

In comparing the serum withdrawn from salvarsan- and neosalvarsan-treated rabbits on successive days after treatment, Stühmer[4]

showed that the duration of the trypanosomicidal action of both was practically the same if the serum was unheated, and that there was but little difference between heated and unheated serum of the neosalvarsan-treated rabbits. The heated serum of salvarsan-treated rabbits, however, was trypanosomicidal several days longer than the same serum not heated. He concluded, therefore, that heating had less effect on neosalvarsanized serum. His method of experimentation did not lend itself to a comparative study of the effect of heating, because he used only one dilution of each serum. His results give rather the duration of trypanosomicidal effect after treatment, and the comparative rate of elimination of salvarsan and neosalvarsan. Experiment 6 (Table VI) showed that neosalvarsan is more active against *Spirochæta duttoni* than salvarsan, but that heating increases the action of both. In each instance the spirocheticidal action of the heated serum was double that of the unheated. Castelli[7] similarly showed that neosalvarsan was more active against *Spirochæta recurrentis* than salvarsan, the ratio being 1:600 to 1:400. The curative dose of both drugs was the same for *Spirochæta gallinarum*, while double the dose of neosalvarsan, compared with salvarsan, was required to cure syphilis in rabbits. Thus the comparative curative dose of the two drugs varies according to the microorganism tested. This experiment proves only that heat had the same relative effect on both salvarsan- and neosalvarsan-serum mixtures.

The proof that a mixture of salvarsan in normal serum had a definite spirocheticidal action suggested that this action might be enhanced if the salvarsan were mixed with the serum of a salvarsan-treated patient. The results of an experiment to determine this point are shown in Table VII. The lethal dose of the salvarsan in normal serum for the spirochete was 0.012 mg. The patient's serum (heated at 56°C.) was completely spirocheticidal in a dilution of 1:1. When half of this dilution was mixed with 0.006 mg. of salvarsan the mixture was completely spirocheticidal, and even when the same amount of salvarsan was mixed with a 1:4 dilution of serum only a slight infection of the mice occurred. Incidentally, the effect of heating salvarsan-treated patient's serum after it has stood 24 hours is well

[7] Castelli, G., *Z. Chemotherap., Orig.*, 1913, i, 321.

TABLE VII.

Effect of Adding Salvarsan to the Serum of a Salvarsan-Treated Patient.

Mouse.	Dilution of serum.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.			
				1	2	3	4
		mg.					
	Normal.						
B3	1 : 1	0.012	Heated.	0	0	Dead.*	–
B4	1 : 1	0.006	"	0	± w	+	0
B5	1 : 1	0.003	"	± w	+	++	0
	Patient's serum.						
E1	1 : 1	0.006	"	0	0	0	0
E2	1 : 2	0.006	"	0	0	0	0
E3	1 : 4	0.006	"	± w	± w	± w	0
G1	1 : 1	None added.	"	0	0	0	0
G2	1 : 2	"	"	±	+	+	0
G3	1 : 4	"	"	+	+++	++	0
H1	1 : 1	"	Unheated.	± w	++	+++	0
H2	1 : 2	"	"	±	+++	++++	0
Control.							
S1	Injected immediately.			+	++++	+	0
S2	1 : 1	After 1 hr. incubation with normal serum.		+	++++	0	0

* Died from mouse typhoid infection.

Dilutions of salvarsan in normal serum were made in the usual way and heated (Series B). A patient with tabes, weighing 70 kilos, received 0.35 gm. of salvarsan intravenously. Blood was withdrawn one-half hour later and allowed to stand over night. It was then centrifugalized and the serum pipetted off, and three series of tubes, E, G, and H, were set up, and the serum was diluted with normal saline solution in Tubes 2 and 3. To each of the tubes of Series E, 0.006 mg. in 0.25 cc. of normal serum was added, after which Series E and G were heated at 56°C. for one-half hour. The spirochetal suspension, containing six to eight spirochetes per field, was added to each tube; it was then incubated for 1 hour and injected into mice.

brought out in the control series, G and H. This experiment seems to prove conclusively that the most potent spirocheticidal mixture with a given amount of salvarsan is to be expected from a combination of salvarsan with the serum of a salvarsan-treated patient, the

blood having been allowed to stand over night before the serum is separated.

DISCUSSION.

Following the introduction of intraspinal injection of serum in the treatment of patients with syphilis of the central nervous system, numerous workers attempted to inject intraspinally salvarsan or neosalvarsan in weak dilutions. When more than 1 mg. has been repeatedly injected several observers have noted symptoms of myelitis in the lower segment of the cord. Marinesco and Minea[8] have observed that neosalvarsan diluted with serum is less irritating than when diluted with normal salt solution. Fordyce[9] and Ogilvie,[2] who have had the greatest experience with the Ogilvie method of preparation of salvarsan serum, now advise that only a fraction of a milligram of salvarsan be mixed with the serum for intraspinal injection. Clinical evidence seems, therefore, to indicate that only small amounts of salvarsan or neosalvarsan can be safely injected into the subdural space. The main objection to subdural therapy of cerebrospinal syphilis has been that the serum injected contained such small amounts of salvarsan. Obviously it is necessary to keep the amount of any therapeutic agent below the injurious dose. Therefore, with the evidence at hand only small amounts of the drugs at our disposal can be used. Our object then must be to use them in the least injurious and most potent form. From the evidence brought forward in the experiments in this communication, this would be the addition of small amounts of salvarsan or neosalvarsan to the serum of salvarsan-treated patients. This serum preferably should be allowed to remain in contact with the clot over night, and should be heated after the addition of the salvarsan. Although this technique is complicated, it could be easily carried out in clinics where large numbers of patients are treated, or if the salvarsan is procurable in ampules containing small amounts, so that dilutions for addition of the drug to the serum might be economically prepared.

[8] Marinesco, G., and Minea, J., *Rev. neurol.*, 1914, xxii, 337.
[9] Fordyce, J. A., *J. Am. Med. Assn.*, 1914, lxiii, 552.

SUMMARY.

1. Addition of salvarsan to serum *in vitro* produces a spirocheticidal mixture which is increased in potency by heating.

2. The heated serum of salvarsan-treated patients is more spirocheticidal if it has been in contact with the clot over night than if it has been separated immediately after coagulation. This is not true with the serum from blood which has been salvarsanized *in vitro*.

3. The addition of salvarsan directly to serum produces a more potent mixture than results from the serum from blood to which salvarsan has been added in equivalent amounts.

4. The increase in activity of salvarsanized serum produced by heating to 56°C. is due in part to the removal of inhibitory substances in the serum and in part to a direct increase in spirocheticidal power in the heated salvarsan.

5. Both salvarsanized and neosalvarsanized serum are rendered more spirocheticidal by heating.

6. A more active spirocheticidal mixture is produced by mixing small amounts of salvarsan with the serum of a salvarsan-treated patient than by mixing the same amount with normal serum.

THE LATENT PERIOD IN THE GROWTH OF BACTERIA.

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The life cycle of a bacterial culture, as determined by estimations of the number of viable organisms present at various intervals, may be divided into four periods or phases, which, in the order of their appearance, may be designated: (1) latent period; (2) period of maximum rate of growth, or logarithmic period; (3) stationary period; (4) period of decline. These phases merge into one another without a sharp dividing line, and their duration varies with different species of organisms, and with the same organism under different conditions, such as temperature, nature of culture media, and still other factors.

By latent period or lag is meant the interval which elapses between the time of seeding and the time at which maximum rate of growth begins. During this time there may be slow growth, no growth, or an actual diminution of viable organisms. The present paper deals with the nature and significance of this phenomenon.

The logarithmic period, so called, is the phase of rapid growth during which the organisms are dividing regularly, so that their increase in a standard unit of time follows the law of geometric proportion, 2 bacteria giving rise to 4, 4 to 8, etc. If, during this period, the logarithms of the numbers of organisms found at varying intervals are plotted as ordinates, against time values as abscissæ, the logarithms fall upon an ascending oblique straight line (1).

This period is followed by the stationary period, in which the organisms cease to multiply at a maximum rate so that their increase in number becomes less and finally ceases; and, although they remain viable, the number present in a unit volume remains approximately constant for an appreciable length of time.

Finally the period of decline sets in, in which the number of living organisms begins to decrease, and at the expiration of several days or longer for certain bacteria living organisms cannot be demonstrated in the culture.

HISTORICAL.

The latent period in bacterial cultures was first recognized by Müller (2) in 1895 in the course of a study of the effect of high temperatures, simulating febrile conditions, upon the growth of the typhoid bacillus. Müller found that the latent period varied with the age of the culture used for seeding, being shorter for younger than for old cultures. He suggested that the latent period results from an alteration of the bacterial cells and that such bacteria transferred to a new favorable medium require time to recover from the injury sustained.

Hehewerth (3) ascertained that the duration of lag is less for *B. coli* than for *B. typhosus*, and shorter when the culture medium is one to which the organism has been accustomed.

Rahn (4) working with *B. fluorescens liquefaciens* studied the influence upon lag of the amount of bacteria used for seeding, and concluded that the larger the inoculum, the shorter the lag. Moreover, he reinoculated bacteria into bouillon from which the previous growth had been removed by filtration through infusorial earth or killed by heat, and found lag still present, although shorter than in fresh bouillon.

Barber (5) studied the rate of multiplication of single individuals of *B. coli* at different temperatures, and found that when three or four generations of bacteria had been formed in a culture, the individual members of that culture failed to show lag upon transplantation. Apparently he was the first to show conclusively that bacteria do not exhibit a latent period under these conditions.

Chick (6) found that *B. coli*, when grown in normal rabbit serum, shows lag, which varies with the temperature at which the organisms are grown, being longer when the temperature is below that of optimum growth.

The latent period has been extensively studied by Penfold (7), who has made an important contribution to the subject. He worked with *B. coli* and determined that with small seedings the lag is longer the smaller the inoculum, but with large ones variations in the size of the inoculum are without effect. He also confirmed Barber's observations, when he showed that subcultures made during the period of maximum rate of growth show no lag. In addition this author found that organisms subjected to a short application of cold during maximal rate of growth cease to multiply and that on reincubation they increase without lag. On the other hand, if the exposure to cold is long, lag appears on reincubation. Efforts to remove lag by washing the bacteria with saline were unsuccessful, as were efforts to demonstrate in the cultures the presence of heat-stable products which affect lag. Finally, he was able to show that organisms

remaining in the supernatant fluid after centrifugation of a culture continue to grow, but with a lag.

Measurement of Lag.—In estimating the rate of growth of bacteria it is convenient to speak in terms of their generation time, the formula for which was first worked out by Buchner, Longard, and Riedlin (8). If the increase always takes place by division of a single bacterium into two new bacterial cells, then, according to the law of geometric proportions $b = a \times 2n$, where b = the number of organisms obtained at the end of a given period of time T, a = the number of organisms present at the beginning of time T, and n = the number of generations which have occurred during the interval. Applying logarithms to this equation, the number of generations, or $n, = \frac{\log b - \log a}{\log 2}$. If there have been n generations in T time, obviously the generation time $G = \frac{T}{n}$.

Hence when multiplication is maximum, the generation time will be shortest, and when it ceases, or an actual decrease in a unit volume occurs, it will be infinity. It follows likewise that the generation time will vary for a single culture, depending upon the particular phase of growth during which observations are made. Consequently, estimations of generation time necessitate frequent observations upon the number of bacteria present; otherwise the results are not of great value.

The duration of lag is measured by the number of minutes or hours which intervene between the time at which the culture is seeded and the time at which maximum rate of growth commences. The onset of the maximum rate of growth corresponds to the minimum generation time.

EXPERIMENTAL.

Technique.—The bacteria employed in this study were *Diplococcus pneumoniæ*, *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens*. The strains of pneumococci used belonged to Types I and II[1] and were laboratory cultures which had been isolated from cases of lobar pneumonia several years previously, but subsequently had been passed through many white mice and transferred innumerable times on artificial media.[2]

The organisms were grown in beef infusion broth, made by extracting a pound of chopped beef with a liter of water over night on ice. The extract was filtered, made up to a concentration of 1 per cent peptone and 0.5 per cent sodium chloride, and boiled ½ hour. The reaction was adjusted to 0.2 per cent acid, using phenolphthalein as an indicator. It was then boiled again for 10 minutes, filtered

[1] See Dochez, A. R., and Gillespie, L. J., A Biologic Classification of Pneumococci by Means of Immunity Reactions, *J. Am. Med. Assn.*, 1913, lxi, 727.

[2] The cultures of *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens* were supplied by Dr. Kligler from the collections of the American Museum of Natural History.

twice through the same paper, and sterilized for 20 minutes on 3 successive days in an Arnold sterilizer. The readiness with which pneumococci grow in the different lots of media prepared in this manner has been found to vary. For this reason, to obtain comparable results with the pneumococcus it is necessary to have a large supply of a given lot of the medium. Results obtained with one lot of broth cannot be compared with those obtained with another lot.

The cultures were incubated in the dark in a water bath at approximately 37°C., in which the variation was always below 1°. Before seeding the broth was brought to 37°C. The number of organisms present in a unit volume was determined by diluting and plating, using 10 to 15 cc. of 1 per cent dextrose agar. The plate method was chosen instead of the direct method of Klein (9) which gives the total number of bacteria, dead and living, since the present study concerns itself especially with the number of living bacteria present in a culture. Dilutions were usually made in normal salt solution, but bouillon was employed when it was desired to observe growth of a few bacteria. Dilutions were carried out by tens, and so arranged that the dilution of the original culture was contained in 0.5 cc. of fluid, the unit volume. Dilutions were made with separate pipettes for each transfer of diluted culture.

During the period of maximum rate of growth of pneumococcus, chain formation is marked, and then diminishes as the age of the culture advances. Hence it might be urged that the latent period demonstrated by the plate method was apparent, rather than real, since a chain as well as a single bacterium may yield only one colony. The tendency of pneumococcus to form chains in fluid media constitutes a real objection to the use of this organism for accurate quantitative work, and renders difficult an estimation of the absolute duration of lag. However, we do not believe that the lag observed in broth culture can be explained on the basis of chain formation alone, for the plate method frequently shows that within the first 2 hours of incubation either no increase or an actual decrease in the number of viable organisms takes place. If the bacteria were multiplying rapidly during this time, although the plates showed only a slight increase or no increase at all, it would be necessary to assume that all or nearly all of the individual cells were forming chains, and that none of these chains were breaking up, an assumption which is scarcely warranted. Next, lag occurs in the case of all motile bacteria thus far investigated in which the question of chain formation can be excluded. Finally, studies which have been carried out in association with Mr. Glenn Cullen, and which will be reported in a subsequent paper, show that the increase in the hydrogen ion concentration of a broth culture of pneumococcus parallels the increase in the number of living bacteria during both the interval of latency and the period of maximum rate of growth, so that there is a lag in the growth of the culture and an equally long lag in the formation of hydrogen ions. The increase in hydrogen ion concentration observed in bacterial cultures indicates that metabolism is taking place, and hence is an expression of change in the medium produced by the agency of the bacteria themselves. It is improbable

that pneumococci carry on extensive metabolic processes independent of growth, since the bacterial cell is a unicellular organism and has a short term of life. Consequently, if, during the latent period no increase in the number of hydrogen ions is observed, it must be assumed that there is little or no metabolism taking place, and hence little or no increase in the number of bacteria.

Phases of Growth of a Bacterial Culture.

Experiment 1.—A flask containing 500 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II. Temperature of water bath 37°C. At frequent intervals a sample was removed for counting. The results are shown in Table I.

TABLE I.

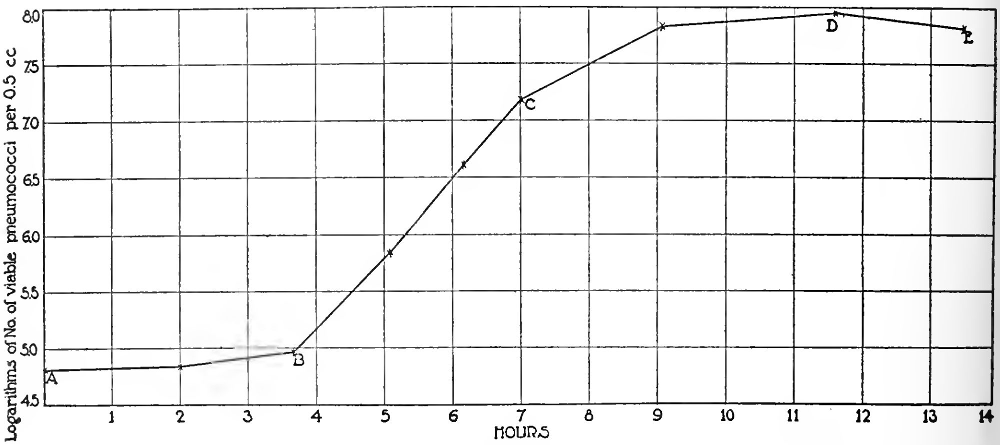
Life Cycle of a Culture of Pneumococcus Type II.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	65,000			
120	70,000	0–120	0.08	1,200.0
220	94,000	120–220	0.42	238.0
305	700,000	220–305	2.9	29.3
370	4,150,000	305–370	2.5	26.0
420	15,200,000	370–420	1.8	27.7
545	67,500,000	220–420	7.3	27.4
695	90,000,000	420–545	2.1	59.0
810	65,000,000	545–695	0.41	366.0
		695–810	0	

The table shows only a slight increase in the number of organisms at the end of 2 hours, and at the end of 3 hours and 40 minutes a single generation has not yet taken place. At the expiration of this period, a rapid increase in the number of organisms begins, which continues almost constant over a period of 200 minutes. These results are brought out more clearly by plotting a curve (Text-fig. 1), employing the time values as abscissæ and the logarithms of the number of organisms in a unit volume as ordinates.

The constant rate of increase during the period of maximum rate of growth is indicated by the fact that the logarithmic values of the number of organisms present fall upon a straight line between

the points *B* and *C*. The period of maximum rate of growth is succeeded by the so called stationary period, during which the growth is less rapid (*C* to *D* in the figure) as shown by the increase in the generation time, and finally the culture reaches the period of decline as indicated by the decrease in the number of viable organisms (from *D* onward). In this particular experiment this stage was reached about 12 hours after seeding. The experiment illustrates the four periods of growth—latent, maximal, stationary, and decline, described at the beginning of the paper. The present study relates to the nature of the first of these periods; namely, the latent period.



TEXT-FIG. 1. Growth curve of a bouillon culture of Pneumococcus Type II, at 37°C.

Latent Period.

Age of Inoculum and Latent Period.—Previous workers demonstrated the influence exerted by certain factors upon the occurrence and duration of this phase. The effect which the age of the culture used for inoculation exerts upon lag has an important bearing upon the nature of the phenomenon. Other investigators in this field all agree that the younger the culture used as inoculum, the shorter the lag. Although both Barber (5) and Penfold(7) have shown that lag is absent in subcultures made when the parent culture is growing rapidly, no attempt has been made to ascertain the particular time in the life cycle of a culture at which subcultures first display lag or

the length of time during which they fail to show it. The following experiments (Nos. 2 and 3), with pneumococcus and *Bacillus coli* as type organisms, show the behavior of subcultures made at frequent intervals during the various stages in the growth of the parent culture. The details of the experiments follow:

Experiment 2.—A flask containing 150 cc. of broth was inoculated with 0.3 cc. of a 24 hour broth culture of Pneumococcus Type I, stock strain, incubated in the water bath at 38°C., and subcultures were made one or more times during its various phases of growth. The growth of both parent culture and subcultures was observed at comparable intervals in each case by the plate method. The results are given in Tables II and III, and are represented in curves shown in Text-fig. 2.

TABLE II.

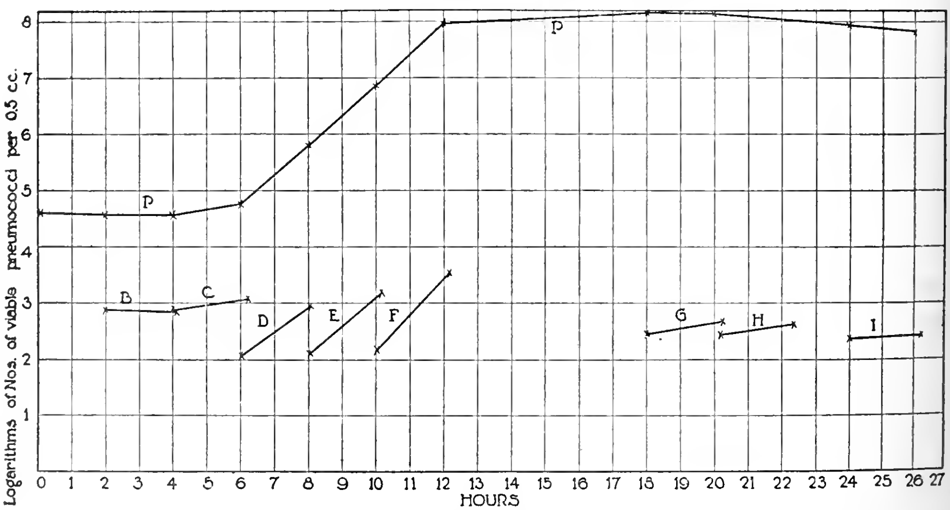
Comparison of Growth in Bouillon of Parent Culture and Subcultures of Pneumococcus Type I.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	41,000			
120	39,000	B	120	785
			245	713
240	38,000	C	240	766
			374	1,200
360	60,000	D	360	120
			485	898
480	625,000	E	480	127
			610	1,580
600	7,400,000	F	600	148
			730	3,490
720	95,000,000			
1,080	145,000,000	G	1,080	290
			1,215	483
1,200	136,000,000	H	1,210	273
			1,340	425
1,440	86,500,000	I	1,440	173
			1,575	263
1,560	65,000,000			

TABLE III.

Comparison of Growth of Parent Culture and Subcultures of Pneumococcus Type I, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Subculture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0– 120	0					
120– 240	0		B	120– 245	0	
240– 360	0.64	187.5	C	240– 374	0.65	206.1
360– 480	3.3	36.3	D	360– 485	2.9	43.1
480– 600	3.9	30.7	E	480– 610	3.6	36.1
600– 720	3.6	33.3	F	600– 730	4.5	28.8
720–1,080	0.6	600.0				
1,080–1,200	0		G	1,080–1,215	0.73	185.0
1,200–1,440	0		H	1,210–1,340	0.63	206.2
1,440–1,560	0		I	1,440–1,575	0.6	225.0



TEXT-FIG. 2. Growth of parent culture and subcultures of Pneumococcus Type I in bouillon at 38° C. *P* indicates parent cultures; *B* to *I* indicate subcultures.

Experiment 3.—A flask containing 50 cc. of bouillon was inoculated with two loops of a 24 hour bouillon culture of *B. coli*, and subcultures were made one or more times during the various phases of growth. The temperature of the water bath was 37°C. The growth of parent culture and subcultures was observed at comparable intervals in each case. The results are given in Tables IV and V and are represented in curves shown in Text-fig. 3.

TABLE IV.

Comparison of Growth in Bouillon of Parent Culture and Subcultures of Bacillus coli.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	20,000			
60	22,000	B	66	431
			129	1,390
120	64,500	C	120	120
			188	843
240	2,150,000	D	240	40
			309	349
			369	2,026
360	86,500,000	E	360	173
			426	614
525	485,000,000	F	525	107
			585	205
			645	1,257
705	970,000,000	G	705	194
			761	331
1,440	1,250,000,000	H	1,440	250
			1,508	378
			1,574	2,060
1,500	975,000,000			

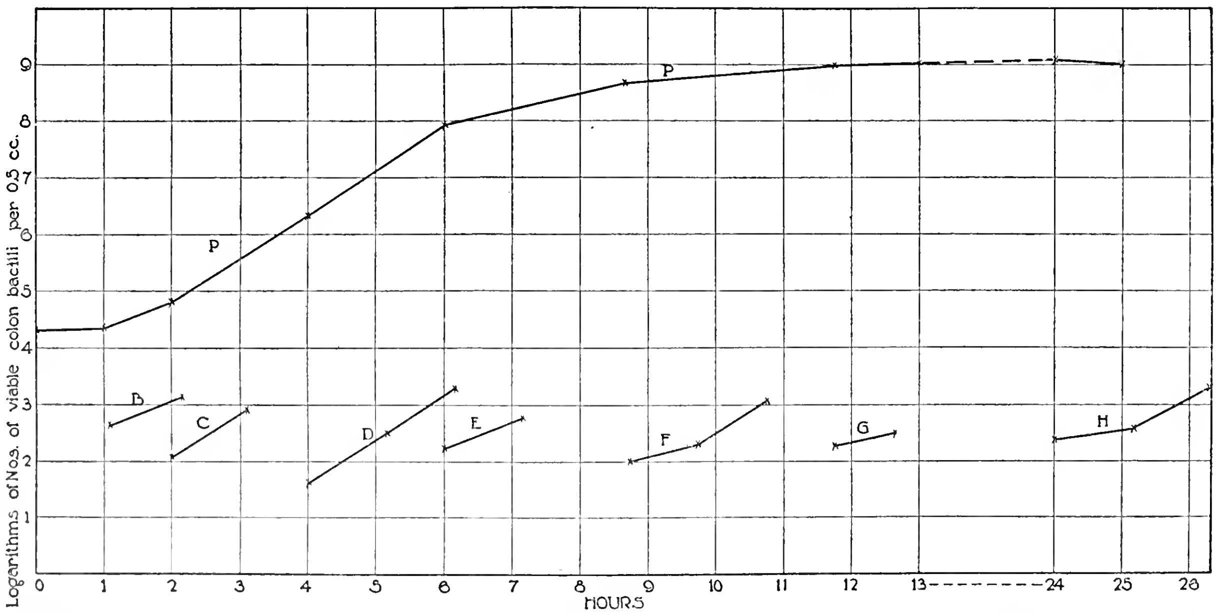
TABLE V.

Comparison of Growth of Parent Culture and Subcultures of Bacillus coli, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Sub-culture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0- 60	0.14	428.5				
60- 120	1.5	40.0	B	66- 129	1.6	39.3
120- 240	5.0	24.0	C	120- 188	2.8	24.2
240- 360	5.3	22.6	D	240- 369	5.6	23.0
360- 525	2.4	68.7	E	360- 426	1.8	36.6
525- 705	1.0	180.0	F	525- 585	0.93	64.5
705-1,440	0.36	2,041.6	G	705- 761	0.77	72.7
1,440-1,500	0		H	1,440-1,508	0.59	115.2

Experiments 2 and 3 show in both instances that if subcultures are made from the parent culture during the period of lag, the subcultures themselves show this same phenomenon. If, on the other hand, the subcultures are made while the parent culture is growing at a constant and maximum rate of growth, the subcultures show no latent period, but grow at approximately the same rate as that of the parent culture. Finally, subcultures made at a time when the parent culture is no longer growing at a maximum rate show a marked lag. This similarity in growth does not obtain if the subcultures are observed over a longer period of time, since after the latent period the subcultures repeat the entire cycle of the parent culture. Other experiments were carried out with *Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus*, and identical results were obtained. They all show the importance of the relationship between the age of a culture and the presence or absence of lag in a subculture. Whether or not lag is present in a subculture depends upon the period in which the culture used for seeding happens to be at the time the subculture is made. That such was found to be the case with four different species of bacteria, motile as well as non-motile, suggests that the fact constitutes a principle applicable to all bacteria.

Occurrence of Lag in the Supernatant Fluid of Broth Culture.—The preceding experiments demonstrate that the behavior of bacteria,



TEXT-FIG. 3. Growth of parent culture and subcultures of *B. coli* in bouillon at 37°C. *P* indicates parent culture; *B* to *H* indicate subcultures.

when transferred to an environment favorable for growth, depends upon that particular period of the life cycle in which the culture happens to be at the time of transfer. The few bacteria remaining in the supernatant fluid after centrifugation of a culture at various phases in its life cycle were next studied to ascertain whether differences occurred in the behavior of the bacteria in the supernatant fluid depending upon the particular time at which the process of centrifugation was carried out.

Penfold (7) conducted one experiment with a 24 hour bouillon culture of *B. coli* and found that the bacteria which were left suspended in the supernatant fluid continued to grow on further incubation but with a definite lag. As no observations were made upon the growth of the culture which he used for centrifugation, it is impossible to say at what phase in its life cycle it happened to be at the time of centrifugation. As the supernatant fluid was kept on ice over night before incubation, a variable factor was introduced, since Penfold has shown that exposure to cold itself suffices to produce lag. No experiments seem to have been made with the organisms remaining in the supernatant fluid after centrifuging a culture during its period of maximum rate of growth.

Experiment 4.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 14 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At three separate periods during the maximum growth of the culture, as indicated in Table VI, 50 cc. were removed and centrifuged at high speed for 30 minutes. Most of the bacteria were thrown down, leaving a clear supernatant fluid containing few organisms. The fluid was transferred to flasks which were incubated in the water bath and estimations of the number of viable organisms present were made at hourly intervals. The results are given in Table VI, and are further illustrated by curves in Text-fig. 4.

TABLE VI.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
60	7,330			
120	11,000	60–120	0.58	103.4
180*	32,500	120–180	1.5	40.0
240	87,000	180–240	1.4	42.8
300*	331,000	240–300	1.9	31.5
360	887,000	300–360	1.4	42.8
420	4,850,000	360–420	2.4	25.0
480*	16,600,000	420–480	1.7	35.2
540	32,900,000	480–540	0.98	61.2
600	39,500,000	540–600	0.26	230.7
660	46,000,000	600–660	0.22	272.7
750	60,000,000	660–750	0.38	236.8

* At this point 50 cc. were removed from the culture and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	4,900			
120	111,000	0–120	4.5	26.6
240	1,300,000	120–240	3.5	34.2

* Obtained by centrifuging the parent culture 180 minutes after the initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	5,800			
120	132,000	0–120	4.5	26.6
240	610,000	120–240	2.2	54.5

* Obtained by centrifuging the parent culture 300 minutes after initial seeding.

TABLE VI—*Concluded.*

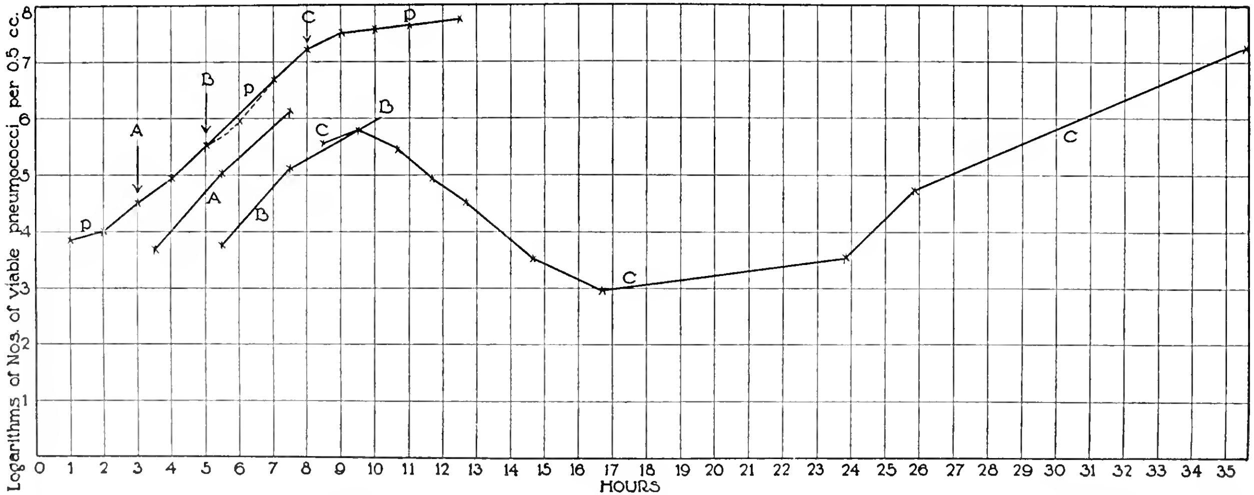
*Growth of Pneumococci in Supernatant C.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	380,000			
60	630,000	0– 60	0.72	83.3
130	300,000	60– 130	0	
190	86,500	130– 190	0	
250	33,000	190– 250	0	
370	3,240	250– 370	0	
490	900	370– 490	0	
920	3,600	490– 920	2.0	215.0
1,040	55,000	920–1,040	3.9	30.7
1,630	17,000,000	1,040–1,630	8.2	71.9

* Obtained by centrifuging the parent culture 480 minutes after initial seeding.

Experiment 4 shows that during the early portion of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to increase in number at approximately the same rate as the organisms in the parent culture. If, however, the centrifugation is carried out toward the end of the period of maximum rate of growth, the viable bacteria in the supernatant fluid decrease in number over an appreciable interval of time (in this case 7 hours), then increase until finally, after 24 hours or more, there are present almost as many viable forms per unit volume as the parent culture attained. These results are illustrated graphically in Text-fig. 4, from which it is seen that the curves of growth of the bacteria in Supernatants A and B, obtained early in the period of maximum rate of growth, parallel the curve of growth of the parent culture; whereas the curve of Supernatant C, obtained at the end of the same period, does not parallel that of the parent culture, but shows, after the lapse of an hour, a fall and a subsequent rise. In other words, the bacteria in Supernatant C exhibit a marked lag. The conclusion to be drawn is that the presence or absence of bacterial lag in a supernatant fluid is dependent upon the age of the parent culture at the time centrifugation is carried out.

It is seen by reference to Text-fig. 4 that while the bacteria are



TEXT-FIG. 4. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A*, *B*, and *C* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

dying the logarithms of the numbers of viable organisms, when plotted against time, fall upon an approximately straight line, indicating that the decrease in viable bacteria is a logarithmic one. It will be of interest to compare the behavior of pneumococci under such conditions with the course of destruction of bacteria when subjected to the action of disinfecting agents.

Krönig and Paul (10) showed that when anthrax spores are killed by disinfecting agents such as mercuric chloride the process is a gradual one. Madsen and Nyman (11) have confirmed these results, and have shown both from their own figures and from those of Krönig and Paul, that the process of disinfection takes place in accordance with a unimolecular reaction. Such a reaction is one in which a substance decomposes in such a manner that the amount undergoing decomposition in a unit of time bears a constant relation to the amount of substance present at the beginning of that interval of time. Chick (12) has shown that the same principle is applicable to the disinfection of anthrax spores and *B. paratyphosus* with phenol. The reaction is represented by the equation $\frac{dN}{dT} = KN$, which on integration yields $K = \frac{1}{t} \log \frac{N_1}{N_2}$ where K is the velocity constant, t is the interval of time between observations, N_1 the number of living bacteria present at the beginning of time t, and N_2 the number present at the end of time t. If the numbers of viable bacteria in a fluid medium decrease in accordance with this law, then the values determined for K should be constant when the values for N_1 and N_2 are substituted in the equation. Furthermore, the logarithms of the numbers of viable bacteria under such circumstances should fall on a descending oblique straight line when plotted against time. Text-fig. 4 shows that the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do decrease for several hours in such a manner that their logarithms fall upon an approximately straight line, which suggests that during this interval they are decreasing in accordance with the law of unimolecular reactions. On this account it seemed advisable to repeat Experiment 4 and obtain more frequent observations with a view of ascertaining whether the equation, which represents the course of such a reaction, can be strictly applied to the figures obtained experimentally.

Experiment 5.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 16 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At two different intervals during the period of maximum rate of growth 50 cc. were removed and centrifuged at high speed for 30 minutes. The supernatant fluid was then removed to a flask and incubated further, and counts of both parent culture and supernatant fluids were made at frequent intervals. The results are given in Table VII and are further illustrated in the form of curves in Text-fig. 5.

TABLE VII.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	13,100			
45	16,200	0– 45	0.3	150.0
185*	140,000	45– 185	3.1	45.1
300	915,000	185– 300	2.7	42.5
425*	11,500,000	300– 425	3.6	34.7
485	19,000,000	425– 485	0.7	85.7
560	39,100,000	485– 560	1.0	75.0
815	61,000,000	560– 815	0.6	425.0
1,220	100,000,000	815–1,220	0.7	578.5
1,575	113,000,000	1,220–1,575	0.17	2,088.2
1,880	80,000,000	1,575–1,880	0	

* At this point 50 cc. of culture were removed and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

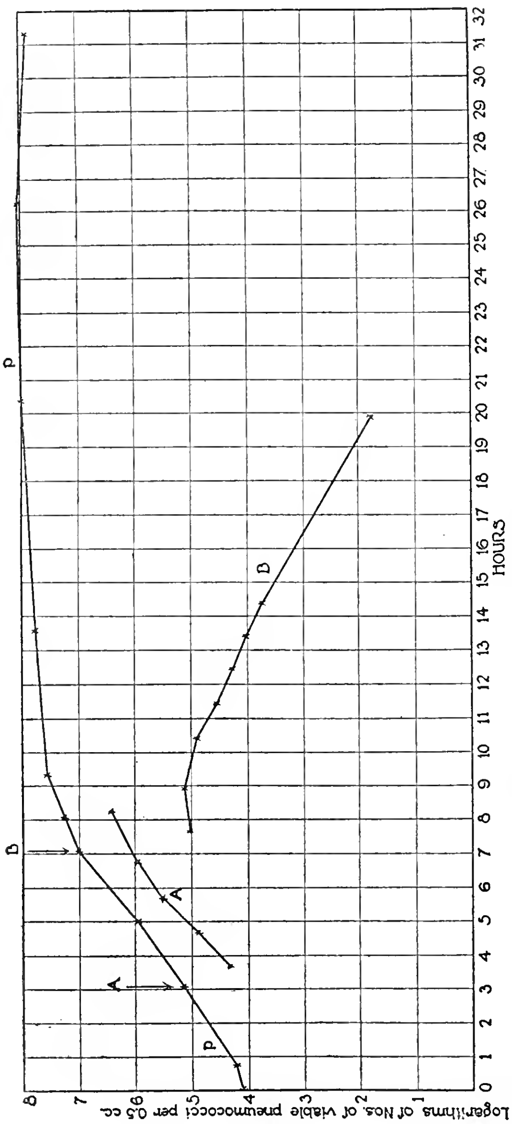
Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	21,300			
60	77,000	0– 60	1.8	33.3
120	335,000	60–120	2.1	28.5
185	885,000	120–185	1.4	46.4
275	2,650,000	185–275	1.5	60.0

* Obtained by centrifuging parent culture 185 minutes after initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	106,000			
75	132,500			
165	80,000			
225	35,000			
285	18,800			
345	10,900			
405	5,400			
735	60			
2,190	Flask cloudy; shows many pneumococci.			

* Obtained by centrifuging parent culture 425 minutes after initial seeding.



TEXT-FIG. 5. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A* and *B* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

In Table VIII are shown the values of K obtained when the numbers of living bacteria in the second supernatant fluid are substituted in the equation $K = \frac{1}{t} \log \frac{N_1}{N_2}$. In this table are also shown the successive values for N_2 obtained by substituting for K its mean value, and for N_1 the number of bacteria found to be present when the organisms had begun to decrease. Opportunity is thus afforded to contrast the calculated theoretical values with those actually found by experimentation. This contrast is also illustrated in Text-fig. 6 in which the theoretical values are plotted in the form of a curve and the results determined by experimentation are designated as dots surrounded by circles.

TABLE VIII.

Application of the Behavior of Pneumococcus Type II in Supernatant B to the Law of Unimolecular Reactions.

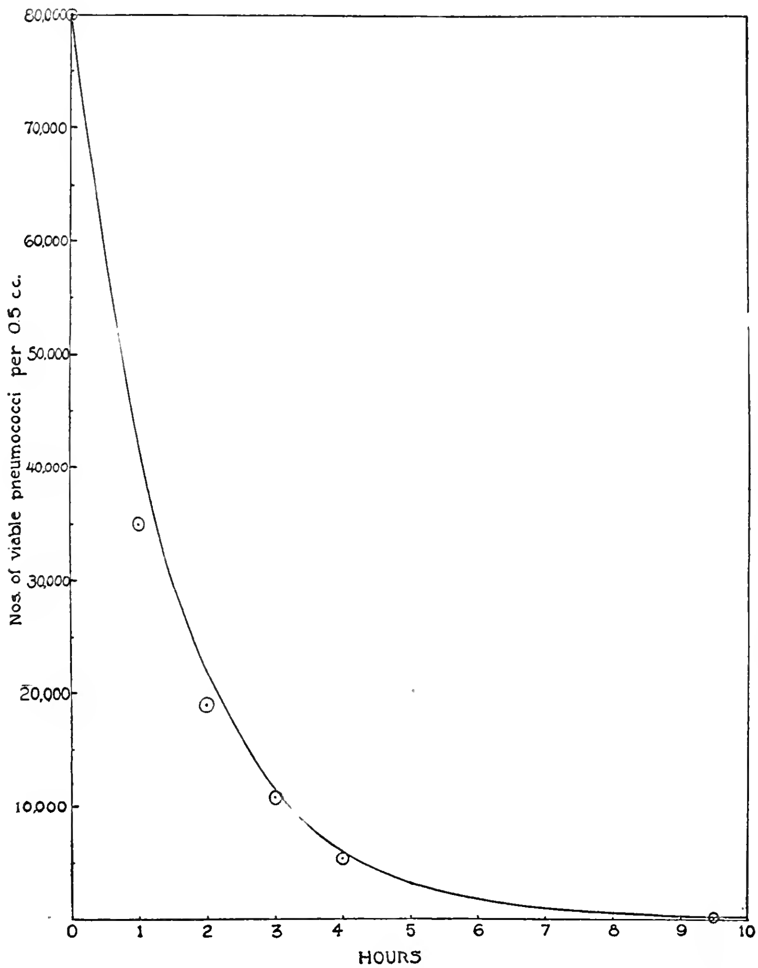
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	Values of K calculated from equation $K=\frac{1}{t}\log\frac{N_1}{N_2}$ assuming $N_1=80{,}000$.	Successive values of N_2 assuming $N_1 = 80{,}000$ and $K = 0.0046$.
min.		*min.*		
625	80,000			
685	35,000	625– 685	0.0056	42,000
745	18,800	685– 745	0.0042	22,000
805	10,900	745– 805	0.0038	11,600
865	5,400	805– 865	0.005	6,100
1,195	60	865–1,195	0.003*	180
2,650	Flask cloudy; shows many pneumococci.			

Mean value of $K = 0.0046$

* The value for the constant K was not calculated when small numbers of bacteria were present, since Chick (12) has shown that in disinfection of *B. paratyphosus* when the culture has reached this stage the velocity of the reaction is slightly greater than one would expect, and the formula cannot be strictly applied.

Experiment 5 confirms Experiment 4 in that it shows that the pneumococci in the supernatant fluid at the beginning of the period of maximum rate of growth continue to multiply at the same rate as those in the parent culture, whereas the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do not continue to grow as do those in the parent culture, but exhibit a marked lag. This experiment shows also, as suggested by Experi-

ment 4, that the bacteria in the second supernatant fluid decrease in number in accordance with the law of unimolecular reactions, since the values obtained for K are quite constant, considering the experi-



TEXT-FIG. 6. Curve showing that the decrease of pneumococci in the supernatant fluid of a broth culture, centrifuged at the end of the period of maximum rate of growth, follows the law of unimolecular reactions. The solid line represents the curve of the calculated values; the dots surrounded by circles represent the values found by experimentation.

mental error necessarily introduced in counting. The closeness with which the course of the destruction of the bacteria follows that of a unimolecular reaction is illustrated by Text-fig. 6 in which the numbers of living bacteria found by experiment fall quite close to or upon the curve which represents the course of such a reaction. It may be said, then, that most of the pneumococci remaining in the supernatant fluid after centrifugation of a culture at the end of the period of maximum rate of growth die within a limited period of time in the course of which they follow the same law as do other bacteria when subjected to the action of disinfecting agents.

The fact that such a close parallelism exists between the behavior of bacteria subjected to the action of disinfectants on the one hand, and the behavior of the pneumococci in the supernatant fluid of broth cultures on the other, suggests that there are present in the supernatant fluid injurious substances that destroy large numbers of the bacteria and arrest the growth of still others. These hypothetical substances must be the products of bacterial growth and demonstrable in every broth culture of pneumococcus. But since the supernatant fluid does not become sterile and ultimately growth is resumed, it follows that either these injurious substances are destroyed or have their effect neutralized, or that the bacteria become adapted to them.

If pneumococci, when grown in broth, form substances inhibitory to their growth, it might be expected that these substances would be present in greatest concentration when the culture had reached the summit of growth. If, however, such substances are unstable on standing at 37°C. as suggested by Experiments 4 and 5, then when a broth culture of pneumococcus is incubated for some period beyond that of maximum growth, they should no longer be present in as great a concentration as that existing at the time when the culture was at its summit. Consequently, if the fluid portion of a culture which has undergone prolonged incubation is reinoculated with the same strain of bacteria, at least partial growth should result, provided the nutritive elements of the medium have not been exhausted. The next experiment was carried out to determine this point.

Experiment 6.—A flask containing 500 cc. of bouillon was inoculated with a culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37°C. for 3 days. Previous experiments (Nos. 1 and 2) have shown that at this time the culture has passed the summit of its growth, and that the bacteria are dying or are nearly all dead. The culture was then centrifuged until a clear supernatant fluid was obtained and this fluid was removed by a pipette to another container. Cultural tests showed only a few viable bacteria. These, however, died off rapidly on further incubation and at the end of 24 hours the fluid was found to be sterile. 50 cc. of this sterile fluid, representing the supernatant of a 4 day culture of pneumococcus, were inoculated with 1 cc. of a culture of the same strain which itself was in the period of maximum rate of growth (7½ hours old). 50 cc. of unused bouillon from the same original lot were inoculated with a like amount of the same culture, thus constituting a control. Both flasks were incubated at 37°C. in the water bath and counts made at frequent intervals. The results are given in Table IX.

TABLE IX.

Comparison of Growth of Pneumococcus Type II in the Supernatant Fluid of a 4 Day Culture of Pneumococcus Type II and in Unused Broth.

Growth in supernatant fluid of 4 day culture.					Growth in unused broth.			
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	4,400				3,000			
120	212,000	0–120	5.5	21.8	110,000	0–120	5.1	23.5
240	2,700,000	120–240	3.6	33.3	925,000	120–240	3.07	39.0
300	5,750,000	240–300	1.09	55.0	2,125,000	240–300	1.2	50.0

Experiment 6 shows that if actively growing pneumococci are reinoculated into bouillon in which the same strain has already grown until the culture has sterilized itself (in this case after incubation at 37°C. for 4 days), they continue to multiply for several hours so that many organisms are formed. If substances are produced in a bouillon culture of pneumococcus which ultimately cause the death of many of the bacteria, then this experiment indicates that after prolonged incubation (in this case 4 days) they are no longer so potent as they were earlier, since they are not able to prevent the formation of several millions of bacteria per unit volume when an actively growing culture of the same strain is reinoculated.

Behavior of Pneumococci in Filtrates of Broth Cultures.—Experiments 4, 5, and 6 suggest that there are formed in broth cultures of pneumococcus substances which have an inhibitory or bactericidal action upon the bacteria themselves and that they lose potency when the cultures are allowed to incubate at 37°C. over a period of several days. In view of these facts, filtrates taken at different periods of growth were tested. Experiments have been reported in the literature which deal with the effect of filtrates of bacterial cultures upon the bacteria themselves when reinoculated into the filtrates. Apparently, however, the question has not been investigated from the standpoint of the relation of the age of a culture to the injurious action of its filtrate. Moreover, in previous work the cultures used for reinoculation have all been of an age sufficient to show a definite lag in the control medium, and for that reason the results are somewhat difficult of interpretation. The following experiment shows that the bactericidal action of a filtrate is dependent in part upon the age of the culture from which it is obtained.

Experiment 7.—A flask containing 1,000 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II, stock strain, and incubated at 37.5°C. At frequent intervals counts were made. At intervals of 1, 2, 4, and 6 days 100 cc. of the culture were removed and filtered through a Berkefeld filter. The same filter was used in each case after careful washing and sterilization. The filtrates were collected separately, and kept on ice until all were assembled and then inoculated with equal amounts of an actively growing (4 hour) culture of the same strain of pneumococcus, and incubated at 37.5°C. Bacterial counts were made at frequent intervals. The results are given in Tables X and XI.

TABLE X.

Growth of the Original Pneumococcus Type II Culture, from Which Definite Amounts Were Removed at Stated Intervals for Filtration.

Time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
hrs.		*hrs.*		*min.*
0	36,600			
1	34,500	0–1	0	
2	36,100	1–2	0.066	909.0
3	41,900	2–3	0.2	300.0
4	57,000	3–4	0.44	136.3
6	310,000	4–6	2.4	50.0
7	710,000	6–7	1.1	54.5
8	2,100,000	7–8	1.5	40.0
9	5,900,000	8–9	1.4	42.8
11.5	145,000,000	9–11.5	4.5	33.3
13	229,000,000	11.5–13	0.6	150.0
14	270,000,000	13–14	0.2	300.0
16	275,000,000	14–16	0.02	6,000.0
21	292,000,000	16–21	0.08	3,750.0
24*	231,000,000	21–24	0	
29.5	140,000,000	24–29.5	0	
48*	38,500,000	29.5–48	0	
75	970,000	48–75	0	
96*	2,280	75–96	0	
144*	0	96–144	0	

* At this point 100 cc. of culture were removed and filtered through a Berkefeld filter, the same filter being used in each case.

TABLE XI.

Comparison of Growth in Filtrates Obtained from a Culture of Pneumococcus Type II at Varying Periods in Its Life Cycle (See Table X).

4 hour culture of same strain of pneumococcus used for inoculation.

Time.	Viable bacteria per 0.5 cc.			
	1 day filtrate.	2 day filtrate.	4 day filtrate.	6 day filtrate.
min.				
0	1,350	1,540	1,380	1,570
60	2,300	6,250	8,800	10,600
120	4,000	11,700	28,700	44,000
180	8,500	19,800	58,000	82,000
245	8,400	28,000	82,000	127,000
330	9,000	34,000	111,000	173,000
420	8,800	46,000	169,000	225,000
515	5,000	39,000	243,000	322,000
605	3,950	35,000	330,000	254,000
695		18,000	363,000	167,000
1,350	8	570	104,000	91,000
	Clear.	Clear.	Slight cloud.	Slight cloud.

The results show that the filtrates obtained at varying intervals after the culture has passed its period of maximum rate of growth, differ in the deleterious effect which they exert upon the growth of the same strain of pneumococcus and that this inhibitory action is most marked at the time when the culture has reached the summit of its growth, after which it becomes progressively less as the culture is allowed to stand at 37.5°C., being least marked when the culture has sterilized itself.

Toxic Substances and Lag.—The experiments show that lag is a phenomenon which bacteria exhibit when the culture used for inoculation has passed the period of maximum rate of growth, and indicate that at this time substances are present in the fluid portion of a culture which exert a deleterious effect upon the bacteria, causing the death of many and temporarily inhibiting the growth of others; furthermore, that these substances are not permanent but disappear or lose their effect in part as the culture becomes older. If this interpretation of the experiments is the correct one, then, by exposing actively growing pneumococci for several hours to the action of a culture filtrate containing these deleterious substances in their maximum concentration, the bacteria should become injured and the injury should be manifested by a failure to grow at a rapid rate immediately upon inoculation into a new medium. In other words, a definite lag should be induced upon otherwise actively growing organisms by this procedure. The exposure would have to be carried out at temperatures below those at which the bacteria grow rapidly, hence in the ice box.

Experiment 8.—A 24 hour bouillon culture of Pneumococcus Type II, stock strain, was filtered through a Berkefeld filter and the filtrate found to be sterile. 1 cc. of a 6 hour broth culture of the same strain was inoculated into 50 cc. of this filtrate and a like amount inoculated into a flask containing 50 cc. of unused bouillon of the same lot as that from which the culture filtrate was obtained. The flasks were immediately placed in the ice chest for 18 hours; their contents were then centrifuged separately at high speed for 20 minutes and the supernatant fluids were removed. The sedimented bacteria were suspended in small amounts of isotonic salt solution and inoculated separately into two flasks containing 50 cc. of unused broth warmed to 37°C. The latter were incubated and the numbers of viable bacteria determined at frequent intervals. The results are given in Table XII.

TABLE XII.

Comparison of Growth of Pneumococcus Type II in Broth, after Exposure to 24 Hour Culture Filtrate and after Exposure to Unused Broth at 0°C. for 18 Hours.

Time.	After exposure to filtrate of 24 hour broth culture.				After exposure to unused broth.			
	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	18,250				7,600			
60	28,200	0– 60	0.62	97.0	19,000	0– 60	1.02	58.0
110	55,000	60–110	0.93	53.0	61,000	60–110	1.6	31.5
180	170,000	110–180	1.6	43.7	312,000	110–180	2.3	30.4
240	409,000	180–240	1.2	50.0	1,070,000	180–240	1.7	35.3

Experiment 8 demonstrates that, by exposing actively growing pneumococci to the action of a culture filtrate of the same strain for a period of 18 hours at 0°C., it is possible to impose upon such bacteria a generation time almost twice as great as that shown by the control organisms. In other words, an increased lag has been induced upon such bacteria by exposing them to an environment in which they have been previously grown.

DISCUSSION.

Several views have been advanced to explain the nature of lag. The fact that bacteria, when inoculated into suitable nutritive media, do not immediately increase at the maximum rate of which they are capable, indicates that there is a lack of complete adjustment between the bacterial cell and its environment, and that this discrepancy must first be corrected before rapid growth can ensue. Obviously, one of two factors, cell or medium, must be at fault. It follows that in order that bacterium and nutritive medium may become completely adjusted to one another a change must take place in one or both of the factors. Lag, then, would represent the time necessary for such a change to be effected.

Keeping these points in mind, the possible causes of lag may be considered under two heads, according to whether the medium or

the bacterial cell is responsible. In other words, the cause may be extracellular or cellular in origin.

Extracellular Theory of the Nature of Lag.—According to this conception, the medium as such is not available for immediate growth and must first be altered by the bacteria themselves in one of the following ways: (1) by the agency of an extracellular enzyme whose function is to split up complex non-available food substances into simpler ones suitable for immediate utilization by the bacterial cell; or (2) by the elaboration of chemical substances which either exert a stimulating effect upon the metabolic processes of the bacteria (as suggested by Rahn (4)) or neutralize preformed toxic substances already present in the medium.

If the causes of lag are extracellular, then lag should always be present no matter what the age of the culture used for inoculation. That such is not the case is demonstrated by the fact that subcultures made from a parent culture when the latter is growing at a rapid rate continue to grow at the same rate and show no lag whatever. This fact, first indicated by Barber (5), later more clearly shown by Penfold (7) to be the case for colon bacilli, and further extended in our experiments with other species, disposes of the extracellular theory of lag and puts the responsibility for failure to grow upon the bacteria themselves.

Cellular Theory of the Nature of Lag.—Since bacterial lag is not due to a fault in the medium, it follows that the cause must be sought in the bacterial cell itself. In Experiments 2 and 3 it was shown that bacteria do not exhibit lag until the culture from which the inoculation has been made has ceased to grow at a maximum rate. When a culture has passed the period of maximum rate of growth the bacteria have changed in some manner since they are no longer able to multiply at once at a rapid rate when transferred to suitable medium. The nature of this change must constitute the cause of lag. The alteration which the bacteria undergo when a culture passes the period of maximum rate of growth may be explained by one or the other of the following reasons. (1) The bacterial cell may lack the proper concentration of intermediate products essential to the synthesis of bacterial protoplasm, a view suggested by Penfold (7); or (2) the bacterial cell may have sustained an injury to those functions or

parts of the cell which are concerned with the maintenance of bacterial metabolism. This conception was suggested by Müller (2) who first described the phenomenon of bacterial lag, although his experiments were not sufficient to establish his view conclusively.

The first idea is not substantiated by the facts brought out in Experiments 4 and 5 which demonstrate that when a broth culture of pneumococcus is centrifuged at the end of the period of maximum rate of growth, the bacteria in the supernatant fluid on further incubation exhibit a marked lag, whereas the bacteria in the original culture continue to grow quite rapidly. Consequently, there is a marked discrepancy in the behavior of the bacteria in the culture as a whole and of those in the supernatant fluid. Why should not the bacteria in each case behave in the same fashion? The lag which is manifested by the pneumococci in the supernatant fluid cannot be due to a lack in those organisms of sufficient intermediate products of metabolism, since the bacteria in the parent culture continue to grow rapidly for several hours at least.

When a culture is centrifuged those bacteria remaining in the supernatant fluid are exposed to the action of all the soluble substances that have been formed as a result of the growth of the culture as a whole. If such metabolic products are toxic for the bacterial cells, it would seem that their action would be stronger when the proportion of metabolic substances to bacterial cells has been increased. It would therefore seem not impossible that the marked lag which is manifested by the pneumococci under these conditions is due to an excess of deleterious substances which cause the death of large numbers and temporarily inhibit the growth of others.

The injurious substances may represent waste products from the cells or unused portions of the molecules of food substances. In any event, the amount present in the fluid is directly proportional to the number of bacteria that have been formed. For that reason they would not be present in any marked amount during the early portion of the period of maximum rate of growth and consequently the bacteria in the supernatant fluid obtained at that time would not show lag. Experiments 4 and 5 show that such is actually the case.

Additional evidence for the view that there are present in the sup-

ernatant fluid obtained at the end of the period of maximum rate of growth substances that exert an injurious effect upon the bacteria is supplied by Experiment 5, which shows that during the time that the organisms in the supernatant fluid are succumbing they do so in accordance with the law of unimolecular reactions, and in this respect their behavior is identical with that of other bacteria when subjected to the action of disinfecting agents.

Although the pneumococci in the supernatant fluid show a marked lag, many of them being killed, the fact that growth is resumed and that ultimately several millions are formed, indicates that the factors responsible for the injurious action have ceased to exist as such. This result may have been brought about through absorption of the substances by the bacterial cells, through spontaneous decomposition, or through decomposition by extracellular enzymes; or, on the other hand, through adaptation of the bacteria to the substances in question.

The idea that the substances are lost through absorption by the cells, or through decomposition or neutralization, is supported by Experiment 6, which shows that after a culture of pneumococcus has sterilized itself through incubation at 37°C. for 4 days, actively growing bacteria when reinoculated continue to grow for several hours. Additional evidence is offered by Experiment 7 which shows that culture filtrates of pneumococci manifest actual loss in inhibitory action, the longer the culture stands at incubator temperatures.

Finally, the view that broth cultures of pneumococcus contain substances that have the property of exerting a direct deleterious effect upon the bacteria themselves is supported by Experiment 8, which demonstrates that it is possible to impose upon actively growing pneumococci an increased generation time by exposing them at ice box temperatures to the action of a filtrate of a 24 hour broth culture of the same strain.

To sum up: (1) Bacterial lag represents an alteration of the bacterial cell. (2) The alteration is concerned with that function or structure of the cell which is essential to metabolism and hence growth of the cell, and is to be regarded in the nature of an injury. (3) The injury is due to the exposure of the cell to direct or indirect products of its own metabolism. (4) The injury may be sufficient

to bring about the death of the cell or it may merely inhibit growth temporarily. (5) The alteration is not always permanent. The bacteria may overcome or survive the condition, or the agents responsible for producing it may suffer actual destruction or be rendered inert. (6) Inasmuch as different media vary in their suitability for bacterial growth, the injury is likely to be most marked when bacteria are grown in a medium which is least favorable.

Stated briefly, it is supposed that if bacteria upon inoculation into suitable media do not begin to multiply immediately at the maximum rate of which they are capable, it is because they have sustained an injury from their former environment.

This view is in accord with the facts elicited by the experiments reported in this paper. It is essentially the view suggested by Müller, although, as already stated, his experimental evidence was meager. It was also advanced by Barber who thought he could distinguish differences in the morphology of the individuals of a 24 hour culture of colon bacilli and those of a culture that was growing rapidly.

The work of Moore (13) lends strong support to this conception of the nature of lag. He has shown that the blood serum of rabbits that have received certain amounts of ethylhydrocuprein (optochin) acquires the property of destroying pneumococci *in vitro*. If smaller amounts of the drug be administered, however, the serum is no longer able to kill the pneumococci, but increases the duration of lag.

The idea that bacteria form toxic substances which in turn inhibit the growth of the organisms is of course not a new one and has given rise to two opposing schools of opinion (14). The evidence here presented indicates that such substances are formed, and that they exert a direct injurious effect upon the microorganisms. Furthermore, these substances appear much earlier than is generally supposed, and on this account it would seem necessary to obtain accurate knowledge of the growth curve of a culture before the terms "old" or "young" are applied to it. Failure to do so has in the past led to erroneous conclusions as to the nature of lag. What is most important to recognize is the fact that the members of a bacterial culture vary considerably in their ability to grow in a given medium, depending upon the particular phase in which the original culture happens to be at the time of transplantation. The bearing

of this fact upon the possibility of variations in virulence of the members of a culture at different stages in its growth is at present under investigation.

Inasmuch as the bacteria in a culture which has passed its period of maximum rate of growth seem to have sustained an injury, it is suggested that in testing the efficacy of any bactericidal agent, actively growing cultures should be used, for if organisms already injured are employed, the result would be a summation of effects due to the agent and those represented by the injury to the bacteria, hence exaggerated ideas might be derived concerning the efficacy of the agent tested. Evidence in favor of this view is offered by the work of Chick (12) who has shown that the individuals of a 3 hour culture of *Bacillus paratyphosus* are more resistant to the action of antiseptics than those of an 18 hour culture of the same strain.

In order to preserve cultures over a long period of time, it has been found best to put on ice those which are in the period of maximum rate of growth, indicated in the case of broth cultures of pneumococcus by the presence of slight turbidity. Such bacteria retain their viability over a longer period than those of 18 hour cultures, hence necessitate less frequent transfers and give greater assurance of recovery, a factor of no little importance in the care of large collections of cultures.

CONCLUSIONS.

1. Cultures of *Diplococcus pneumoniæ*, *Bacillus coli*, *Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus*, when grown in meat infusion broth exhibit an initial latent period when the culture used for inoculation is no longer growing at its maximum rate; if, however, the culture is growing at its most rapid rate the bacteria, upon subculture, show no latent period but continue to multiply at the same rate as that of the parent culture.

2. If broth cultures of pneumococcus are centrifuged at the beginning of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to grow at a rapid rate upon further incubation; if, however, the culture is centrifuged at the end of the period of maximum rate of growth, those bacteria which remain in the supernatant fluid show a prolonged latent period, during

which many of the organisms die. While the death of these bacteria is taking place the process follows closely the law of unimolecular reactions.

3. Actively growing pneumococci inoculated into the supernatant fluid from a 4 day culture of the same strain continue to grow rapidly for an appreciable time after inoculation.

4. Filtrates from 24 hour cultures of pneumococcus inhibit the further growth of actively growing pneumococci when the latter are inoculated into such filtrates. This inhibitory action of the filtrates is lost in part as the culture from which the filtrate is obtained is allowed to incubate longer.

5. Actively growing pneumococci, after exposure at low temperatures to the action of the filtrate of a 24 hour broth culture of the same strain, show a greater lag than the controls.

6. The foregoing facts offer strong support for the view that lag is an expression of injury which the bacterial cell has sustained from its previous environment.

BIBLIOGRAPHY.

1. Lane-Claypon, J. E., *J. Hyg.*, 1909, ix, 239.
2. Müller, M., *Z. Hyg. u. Infectionskrankh.*, 1895, xx, 245.
3. Hehewerth, F. H., *Arch. Hyg.*, 1901, xxxix, 321.
4. Rahn, O., *Centr. Bakteriol., 2te Abt.*, 1906, xvi, 417.
5. Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.
6. Chick, H., *J. Hyg.*, 1912, xii, 414.
7. Penfold, W. J., *J. Hyg.*, 1914, xiv, 215.
8. Buchner, H., Longard, K., and Riedlin, G., *Centr. Bakteriol., 1te Abt.*, 1887, ii, 1.
9. Klein, A., *Arch. Hyg.*, 1902, xlv, 117.
10. Krönig, B., and Paul, T., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 1.
11. Madsen, T., and Nyman, M., *Z. Hyg. u. Infectionskrankh.*, 1907, lvii, 388.
12. Chick, *J. Hyg.*, 1908, viii, 92.
13. Moore, H. F., *J. Exp. Med.*, 1915, xxii, 551.
14. Kruse, W., Allgemeine Mikrobiologie, die Lehre vom Stoff- und Kraftwechsel der Kleinwesen, für Aerzte und Naturforscher dargestellt, Leipsig, 1910, 160.

A RAPID METHOD FOR THE DIAGNOSIS OF RENAL TUBERCULOSIS BY THE USE OF THE X-RAYED GUINEA PIG.

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The value of animal inoculation in the diagnosis of tuberculosis has been recognized since Marcet's[1] experiments in 1867. Klebs[2] confirmed this work in the following year, and the constant employment of the guinea pig test since that time has proved it to be the safest diagnostic method that we have.

It is natural that this method should be given special importance in cases of renal tuberculosis where there is always the complicating smegma bacillus to rule out on microscopic examination, and where the demonstration of the tubercle bacillus is a matter of considerable difficulty. In the past year Crabtree[3] published his technique for high centrifugalization of the urine, an important step forward, but in the same article he admits that guinea pig inoculation must be the final test in all doubtful cases.

Damsche[4] was the first investigator to use the guinea pig for the diagnosis of renal tuberculosis. From his time to the present there has been little change in the technique employed. In the Massachusetts General Hospital a subcutaneous injection of 15 to 20 minims of suspected urine is made into the abdominal wall of the animal and after 5 weeks an autopsy is done. The diagnosis is made both by gross lesions and by microscopic examination of film preparations. Barney and Young's[5] summary of 197 cases from this hospital shows

[1] Marcet, W., *Tr. Med. Clin.*, 1867, 437.

[2] Klebs, E., *Virchows Arch. path. Anat.*, 1868, xliv, 242, 296.

[3] Crabtree, E. G., *Surg., Gyn. and Obst.*, 1916, xxii, 221.

[4] Damsche, *Deutsch. Arch. klin. Med.*, 1882, xxxi, 78 ff.

[5] Barney, J. D., and Young, E. L., Jr., *Boston Med. and Surg. J.*, 1911, clxiv, 917.

that the reports previous to operation have been correct in 99.5 per cent of the cases.

The method is, however, open to criticism on account of the time that is lost while waiting for the lesions to develop in animals.[6] Any modification, therefore, that will allow us to shorten the time appreciably should be of practical value. With this in mind, the following experiments were undertaken.

Murphy and Ellis[7] have shown that white mice which have been exposed to x-rays are made markedly more susceptible to bovine tuberculosis than normal animals. They explain this fact by the destruction of the lymphoid tissue which they claim constitutes an important agent in the defensive mechanism against tuberculosis. It seemed reasonable to assume that the guinea pig could be rendered less resistant than it is normally by a similar procedure. Accordingly, guinea pigs were given various doses of Roentgen rays to determine how much they could stand and whether the resistance to known tuberculous urine could be reduced.

It was found that guinea pigs could tolerate a large amount of x-rays without apparent injury to their health. The average normal white blood count of the animals was from 12,000 to 15,000 cells per c. mm., of which 40 to 45 per cent were of the lymphocytic variety, and, with the exception of ½ to 1 per cent of transitionals and large mononuclears combined, the remaining cells were polymorphonuclears. One massive treatment with x-ray sufficed to reduce the total count by over one-half (4,000 to 6,000), mainly effected by reduction in the lymphoid cells. The count remained depressed for a period of over 1 week, which was as far as it was followed. Animals treated in this way when inoculated with a known tuberculous urine developed marked lesions of the disease in a much shorter time than did normal animals. Further experiments showed that an x-ray exposure given shortly after inoculation was as effective in reducing the resistance as when given before the inoculation. This fact seems to be of practical importance as it obviates the necessity of keeping a stock of treated animals on hand. After having established a working dosage and the best time interval before killing the animals, a series of cases was studied in connection with the Genito-Urinary

[6] Churchman, J. W., *Med. Rec.*, 1916, lxxxix, 511.

[7] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

Department of the Massachusetts General Hospital, in order to test the usefulness of the method.

It was considered necessary for practical reasons to confine the x-ray preparation to one exposure only, sufficiently massive to carry the animals through 10 days with depressed resistance. It is probable, however, as is shown by the experiments of Murphy and Morton,[8] that repeated small exposures would be more satisfactory for experimental study when it is desirable to produce a maximum effect on the lymphoid tissue with a minimum damage to other structures. The following dosage was adopted as the most useful. The animal was placed in a pasteboard box of such a size that it could not move around, and then radiated for a period of 10 minutes with the Coolidge tube, the target being 12 inches distant from the base of the box. A 5 milliampere current was passed through the tube, backing up $8\frac{1}{2}$ inches of spark between points. The Snook interrupterless was the type of machine employed. No aluminum or leather filters were used. From 1 to 2 cc. of urine were injected intraperitoneally according to the amount at hand. After 10 days the guinea pigs were killed and examined for the lesions of tuberculosis. As in the normal animals, the usual sites of election were the spleen, mesenteric lymph glands, and liver. The lesions varied with the severity of the infection,—sometimes the spleen was riddled with a mass of caseous nodules 2 to 4 mm. in diameter, while at other times the spots were more nearly the size of a pinhead but distinctly pathognomonic. The lymph glands were usually from 1 to 1.5 cm. in diameter and caseous, and, as a rule, the liver had scattered over its surface nodes similar in size to those of the spleen. Films taken from these situations and stained for tubercle bacilli were used to confirm the gross diagnosis. Microscopically the picture was one of coagulation necrosis and caseation with a zone of endothelioid cells but no formation of giant cells, and a striking absence of the small round cell infiltration. There was also no evidence of an enclosing fibrous layer such as is commonly seen in the slow growing tubercle.

The following cases were taken at different times from the Genito-Urinary Department, and the above method of making the diagnosis was used. The guinea pig findings were confirmed in every case as far as possible by all other data which would tend to throw light on possible error. The results are given in Table I.

[8] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

TABLE I.

Case No.	Urine.	Cystoscopy.	Normal guinea pig test.	X-rayed guinea pig test.	Operation.	Pathological report.	Clinical diagnosis.	Remarks.
1	Large numbers of tubercle bacilli in clumps. Culture not done.	Tuberculous bladder.	1. Killed in 5 weeks. Spleen full of nodules of tubercle bacilli. 2. Died during night.	Both animals died on following day.	None.	None.	Tuberculosis of both kidneys and of lungs.	One normal and two x-rayed animals died in 24 hrs. from a mixed streptococcus infection. One normal animal showed tuberculous lesions of spleen after 5 weeks.
2	Sediment, from left kidney showed tubercle bacilli; from right kidney few tubercle bacilli. Culture, no growth.	Tuberculous pus in left ureter. No pus in right ureter.	No test.	Killed on 10th day. Liver covered with patches. Spleen contained small nodules. Glands caseous.	"	"	Tuberculosis of left kidney. Tuberculosis of right kidney? Tuberculosis of lungs.	Question of right kidney excreting bacilli without disease. No pus in urine on right side. Tubercle bacilli in sputum.
3	Sediment, from left kidney showed few tubercle bacilli; from right showed tubercle bacilli. Culture, few cocci from left; no growth from right.	"Not diagnostic."	Not yet reported.	Killed on 7th day. Extensive lesions in liver, spleen, and glands.	"	"	Tuberculosis of both kidneys.	
4	Sediment, many tubercle bacilli. Culture not made.	Not done.	No test.	Extensive lesions in liver, spleen, and lymph glands.	Nephrectomy.	Tuberculosis of kidney.	Tuberculosis of kidney.	

5	Sediment, few tubercle bacilli after high centrifuge. Culture, no growth.	Could not catheterize.	"	Killed on 10th day. Spleen riddled. Glands caseous; size of 10 cent piece.	"	"	Tuberculosis of kidney or bladder.	
6	Sediment, negative for tubercle bacilli. Culture, no growth.	Nothing suggestive.	Positive for tubercle bacilli in 5 weeks.	Killed on 9th day. Spleen and lymphoid glands caseous.	"	"	Tuberculosis of kidney.	Two x-rayed guinea pigs used. First killed on 9th day with diagnostic lesions; second killed in 5th week with severe lesions.
7	Sediment, negative for tubercle bacilli. Culture, no growth.	Left ureter retracted. Tuberculosis?	Negative four times.	Killed on 8th day. Liver peppered with pin-point lesions. Spleen also involved.	"	"	Tuberculosis of kidney.	Four normal guinea pigs gave negative results.
8	Sediment, colon bacilli. Culture not made.	Ulcer near right ureter, tuberculous.	No test.	Killed on 14th day. Liver and spleen contained pin-point caseous nodules.	"	"	Tuberculosis of right kidney.	
9	Sediment, no bacilli. Culture not made.	Suggests bilateral tuberculosis.	"	Died on 8th day. Peritonitis. Film showed tubercle bacilli present.	"	"	Tuberculosis of left kidney. Pott's spine. General peritonitis.	Mixed infection with doubtful lesions.
10	Sediment, negative for tubercle bacilli. Culture, streptococci.	Not done.	"	Killed on 14th day. Marked lesions in liver. Spleen riddled. Glands caseous.	None.	None.	Tuberculous epididymitis. Tuberculosis of bladder or kidney?	

TABLE II.

Case No.	Urine.	Cystoscopy.	Normal guinea pig test.	X-rayed guinea pig test.	Operation.	Pathological report.	Clinical diagnosis.	Remarks.
1	Sediment, no tubercle bacilli. Culture, staphylococci.	Normal.	Negative for tubercle bacilli.	Killed on 14th day. Marked lesions in liver and spleen.	None.	None.	Pyonephrosis or pyelitis.	Film cleared diagnosis. Cocci. Negative for tubercle bacilli.
2	Sediment, no tubercle bacilli; colon bacilli. Culture, colon bacilli.	"Bladder looks like subsiding colon infection."	No test.	Killed on 12th day. Plastic peritonitis. No tuberculosis.	"	"	Cystitis.	
3	Sediment, no tubercle bacilli. Culture, staphylococci.	"Practically normal."	Negative for tubercle bacilli.	Killed on 10th day. No lesions.	"	"	"	
4	Sediment, no tubercle bacilli. Culture, staphylococci.	Impossible.	"	Killed on 10th day. No lesions.	"	"	Cystitis. Renal stone.	
5	Sediment, no tubercle bacilli. Culture, staphylococci.	Not done.	"	Killed on 10th day. General peritonitis.	"	"	Cystitis.	
6	Sediment, no tubercle bacilli. Culture, colon-bacilli.	"Suggestive of tuberculosis."	"	Killed on 10th day. Foul general peritonitis. Culture, staphylococci.	"	"	Pyonephrosis. Cystitis.	

7	Sediment, no tubercle bacilli. Culture, colon bacilli.	Not done.	"	Killed on 10th day. Urine from left and right ureters negative.	"	"	Cystitis.	
8	Sediment, no tubercle bacilli. Culture, staphylococci.	"	No test.	Died on 7th day. General peritonitis. No tuberculosis.	"	"	"	
9	Sediment, no tubercle bacilli. Culture, streptococci.	Irritable bladder, small capacity. Could not catheterize ureter.	"	Killed on 10th day. Bladder urine gave no lesions.	"	"	"	
10	Sediment, left, few cocci; right, no tubercle bacilli. Culture, left, staphylococci; right, no growth.	Normal.	Negative for tubercle bacilli.	Killed on 10th day. Urine from left ureter gave small lesions. Film, cocci. Urine from right ureter negative.	"	"	Pyelitis.	Film rules out atypical lesions. No tubercle bacilli.

Cases 1, 2, and 3 were extensive renal tuberculosis, bilateral, and of course inoperable. They were used early in the series and are included to make the gradations of severity complete. Case 1 is instructive in that the x-rayed guinea pigs and one of the normal animals were killed by a mixed streptococcus infection. The other normal guinea pig survived, and when it was killed after 5 weeks, the spleen was full of tuberculous nodules. Case 2 presents an interesting possibility; namely, that the left kidney alone was involved, and the right was excreting tubercle bacilli without being affected. That this does occur at times is recognized in the literature (Brown[9]). Cases 4 and 5 were moderately severe and the bacilli were found in the urine by use of the high centrifuge method. Cases 6 to 10 were not advanced cases and no tubercle bacilli were demonstrated by microscopic examination of the sedimented specimens. Case 7 was tried four times in normal guinea pigs with negative results, probably because the ureter was occluded. On the first trial with the x-rayed guinea pig the test was successful in 8 days, an example of good fortune in getting a specimen which contained the bacilli. Case 9 had a mixed infection and the lesions were atypical. Acid-fast bacilli were demonstrated in films made from the animal, but the report would not have been made as positive without repetition except for the evidence of Pott's disease of the spine in this case. Most of these cases were reported within the 10 day limit, but three of the earlier ones were allowed to go 2 weeks before a diagnosis was made. These three cases would have undoubtedly fallen into the same period as the others, as the lesions were extensive in each instance.

A table of the non-tuberculous conditions in which it was necessary to rule out tuberculosis is also presented (Table II).

In these ten cases the diagnosis "negative for tuberculosis" has been confirmed by the clinical course, by tests with normal guinea pigs, and by all other methods available. Cases 1 and 10 (same individual) were the only ones which gave lesions in any way comparable to tuberculosis. In these cases there were multiple abscesses which in films showed no tubercle bacilli, but many small micrococci. Other guinea pigs in the series died early of plastic peritonitis from which pure cultures of the organism responsible were recovered.

[9] Brown, L., *J. Am. Med. Assn.*, 1915, lxiv, 886.

DISCUSSION.

While the number of cases here presented is comparatively small, the uniformity of the results justifies a report at this time. The work is of value from the experimental side in that it confirms and extends the observation of Murphy and Ellis, in which they showed that white mice were made markedly more susceptible to bovine tuberculosis by suitable x-ray exposures. These experiments demonstrate that the same conditions hold for the human strain of the organism in the guinea pig. As to whether this lowered resistance is due to the destruction of the lymphoid elements as is suggested by Murphy and Ellis, the present work is perhaps not conclusive. For a demonstration of this point it would be better to use the fractional dose method as recommended by Murphy where a maximum effect may be obtained on the lymphoid elements with a minimum on the other structures. For obvious reasons, however, this method would not be practical for clinical use.

SUMMARY.

It is possible to reduce the resistance of guinea pigs to tuberculosis by x-ray exposures, so that when animal inoculation is required for a diagnosis a much quicker result may be had than by the use of normal animals. In renal tuberculosis when it is necessary to resort to the use of animals, it ordinarily requires from 5 to 7 weeks, while by the use of x-rayed guinea pigs the diagnosis can be made in from 8 to 10 days. The resistance can be sufficiently lowered by one massive dose of x-ray administered either shortly before or after the inoculation of the material to be tested. The lesions are so marked in these animals that the diagnosis is certain after the interval indicated above.

I wish to thank the members of the Staff of the Genito-Urinary Department for the interest they have taken and the assistance they have rendered in making this work possible.

429,

CICATRIZATION OF WOUNDS.

I. The Relation between the Size of a Wound and the Rate of Its Cicatrization.

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In the course of experiments made in 1908 at The Rockefeller Institute certain relations existing between the size of a wound and the rate of cicatrization were studied. The experiments showed that the rate of repair was greater at the beginning than at the end of cicatrization, and depended not on the age of the wound but on its size, being directly proportional to it.[1] The skin of mammals seemed therefore to regenerate according to a law similar to that found by Spallanzani for salamanders. The experiments made lately by Spain and Loeb showed that in the guinea pig the same relation existed between the size of a wound and the rate of repair.[2] The object of the following experiments was to find a technique by which the size of a wound could be measured accurately, to ascertain whether the curve representing the cicatrization was geometric in form, and to study the relations between the size of a wound and the velocity of repair, as well as the relative importance of the processes of contraction and epidermization.

EXPERIMENTAL.

The experiments were made in the following manner. In the sternal region or in the anterior abdominal region of anesthetized guinea pigs and cats, wounds were obtained by resection of a strip of skin geometric in form. In order that the edges of the cicatrix

[1] Carrel, A., *J. Am. Med. Assn.*, 1910, lv, 2148.

[2] Spain, K. C., and Loeb, L., *J. Exp. Med.*, 1916, xxiii, 107.

might be seen distinctly, animals with a black skin were used, or the edges of the wound made on white animals were tatooed with India ink. The skin of the cat and, as had been previously noted, of the dog not being adherent to the aponeurosis, errors occurred in the measurement, if, in consecutive observations, the animal was not placed in an identical position. The guinea pig was generally employed because the skin of the abdominal wall of this animal is more adherent to the aponeurosis than that of the cat or dog. In human beings wounds of regular shape were selected, located on patients confined to bed. When both the wound and the cicatrix were to be studied, cases were chosen in which the outer edge was well colored and easily discernible from the surrounding skin. Observations were also made on the healing wounds of soldiers.

During the period of observation the wounds were kept by means of antiseptic and aseptic dressings in as constant a bacteriological condition as possible. Every day films of the secretions, taken in different parts of the wounds, were examined. When the films contained bacteria the wound and the surrounding skin were cleaned with a cotton sponge and neutral sodium oleate. Then, for a few hours, 0.5 per cent of Dakin's hypochlorite or 1 per cent paratoluene sodium sulphochloramide was instilled on the wound. As soon as the bacteria had disappeared, aseptic dressings or compresses moistened with 0.2 per cent paratoluene sodium sulphochloramide were applied. The wound remained aseptic for several days. If bacteria appeared again on the granulating surface, chemical sterilization was used.

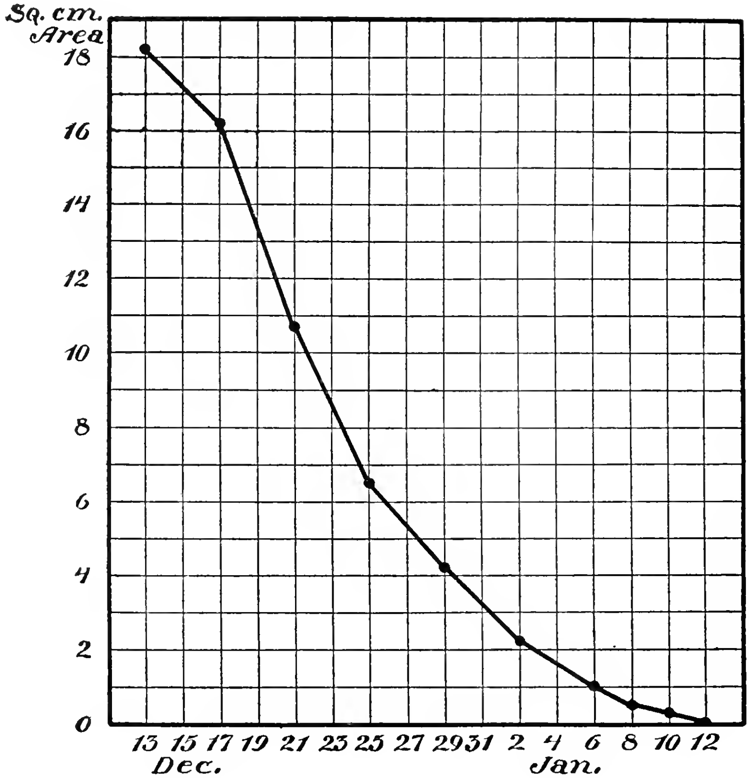
The measurements of the wound were taken at regular intervals, as a rule every 4 days. The procedure was as follows: After the surface was thoroughly dried by antiseptic gauze or by filter paper, a sheet of thin, transparent cellophane was applied with a sponge directly over the granulations and the epithelial edge. The inner edge of the epithelium could then be clearly discerned, and was even felt under the point of the wax pencil by means of which it was drawn on the cellophane. The outer edge of the pencil mark corresponded to the line of union between the epithelium and the granulating surface. Two drawings were made at each examination. The outer edge of the cicatrix was also outlined. The drawings obtained on

cellophane were then reproduced in ink on a sheet of paper. This second drawing was used for the measurements. By means of a planimeter the area of the wound (S) and the area of the wound and the cicatrix ($S + C$) were estimated in square centimeters. The daily rate (R) of cicatrization was obtained by dividing the differences of two consecutive surfaces by the time which had elapsed between the two observations. The value of the rate of cicatrization in its relation to the area was obtained by the formula $\frac{S}{V}$.

Through the values of S and R a curve was established in which the time was carried in abscissæ, and the area in ordinates. The tracing representing the variations of the surface located within the outer edge of the cicatrix was drawn above the curve of the surface of the wound. The interval between both curves represented the area of the cicatrix in square centimeters. Thus it was possible to ascertain daily, in square centimeters, the size of the wound, the size of the cicatrix, and the velocity of the process of repair. By means of this technique, the relations which exist between the size of a wound and the rate of cicatrization, and the respective value of contraction and of epidermization in the process of repair, were studied.

The Relation between the Size of a Wound and the Velocity of Repair.

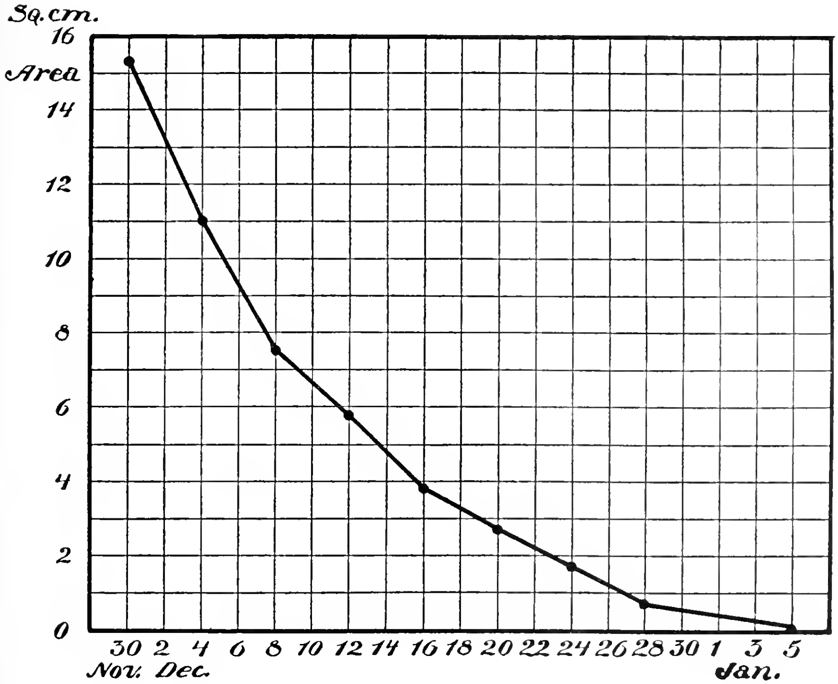
The observations were made on men and on guinea pigs. The wounds were in a condition of slight infection and healed both by contraction and by epidermization.



TEXT-FIG. 1.

Experiment 1.—Patient 221, age 27 years (Text-fig. 1). Old wound of the left foot; aseptic. Normal cicatrization.

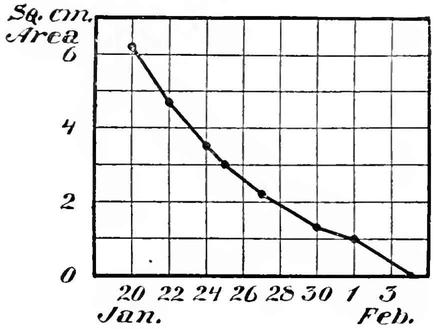
	Dec. 13	17	21	25	29	Jan. 2	6	8	10	12
S........	18.2	16.2	10.7	6.5	4.2	2.2	1.0	0.5	0.3	0
R........		0.5	1.37	1.05	0.55	0.5	0.3	0.25	0.1	0.15
$\frac{S}{R}$........	36.0	11.8	10.1	11.8	8.4	7.3	3.0	5.0	2.0	



TEXT-FIG. 2.

Experiment 2.—Patient 217, age 36 years (Text-fig. 2). Wound of the left iliac region; slightly infected. The appearance of the curve is normal although the rate of repair was slow.

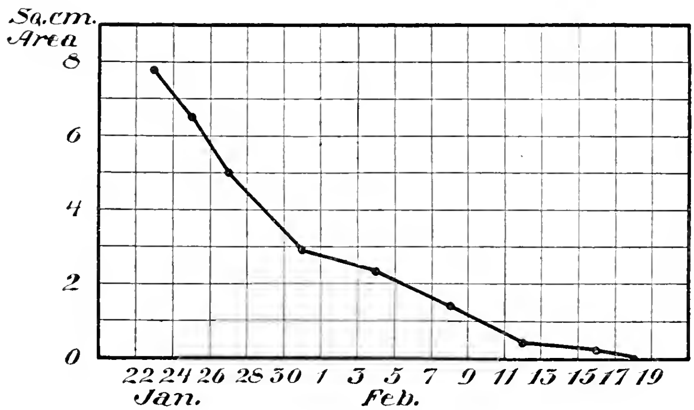
	Nov. 30	Dec. 4	8	12	16	20	24	28	Jan.
S...............	15.3	11.0	7.5	5.8	3.8	2.7	1.7	0.7	0
R...............		1.07	0.87	0.42	0.5	0.17	0.25	0.25	0.1
$\frac{S}{R}$...............	14.3	12.6	17.8	11.6	22.0	10.8	6.8	7.0	



TEXT-FIG. 3.

Experiment 3.—Patient 354, age 40 years (Text-fig. 3). Old wound on a stump; slightly infected. Normal cicatrization. The irregularity of the curve on Feb. 1 was due to the dressing.

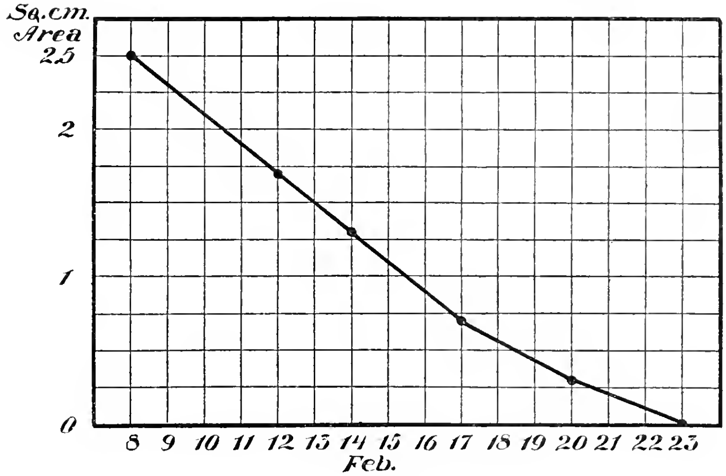
	Jan. 20	22	24	25	27	30	Feb. 1	4
S........................	6.2	4.7	3.5	3.0	2.2	1.3	1.0	0
R........................		0.75	0.60	0.50	0.40	0.3	0.15	0.33



TEXT-FIG. 4.

Experiment 4.—Patient 330, age 32 years (Text-fig. 4). Wound following the amputation of the index finger and of a part of the metacarpus; slightly infected. From Jan. 31 the rate of cicatrization decreased on account of infection.

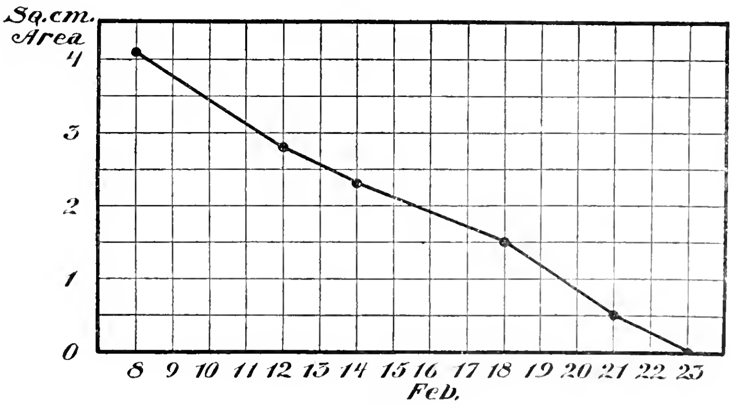
	Jan. 23	25	27	31	Feb. 4	8	12	16	18
S................	7.8	6.5	5.0	2.9	2.3	1.4	0.4	0.2	0
R................		0.65	0.72	0.52	0.15	0.22	0.25	0.05	0



TEXT-FIG. 5.

Experiment 5.—Guinea Pig 1 (Text-fig. 5). Wound of the anterior abdominal wall; slightly infected. Normal cicatrization.

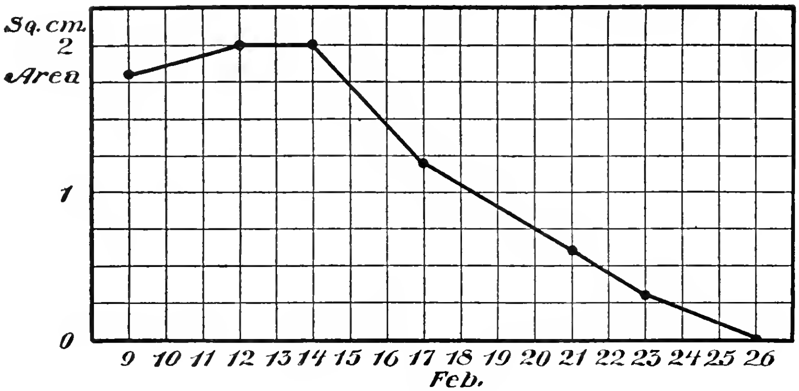
	Feb. 8	12	14	17	20	23
S..................................	2.5	1.7	1.3	0.7	0.3	0
R..................................		0.8	0.2	0.2	0.15	



TEXT-FIG. 6.

Experiment 6.—Guinea Pig 2 (Text-fig. 6). Wound of the anterior abdominal wall. The curve is nearly regular in spite of a slight infection. The slackening of the rate of cicatrization on Feb. 18 was due to a more marked condition of infection.

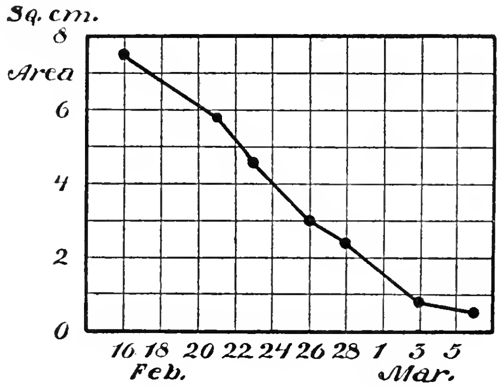
	Feb. 8	12	14	18	21	23
S..................................	4.1	2.8	2.3	1.5	0.5	0
R..................................		0.32	0.25	0.20	0.3	



TEXT-FIG. 7.

Experiment 7.—Guinea Pig 3 (Text-fig. 7). Wound of the anterior abdominal wall; slightly infected. The horizontal part of the curve from Feb. 9 to 14 represents the period normally preceding the beginning of contraction.

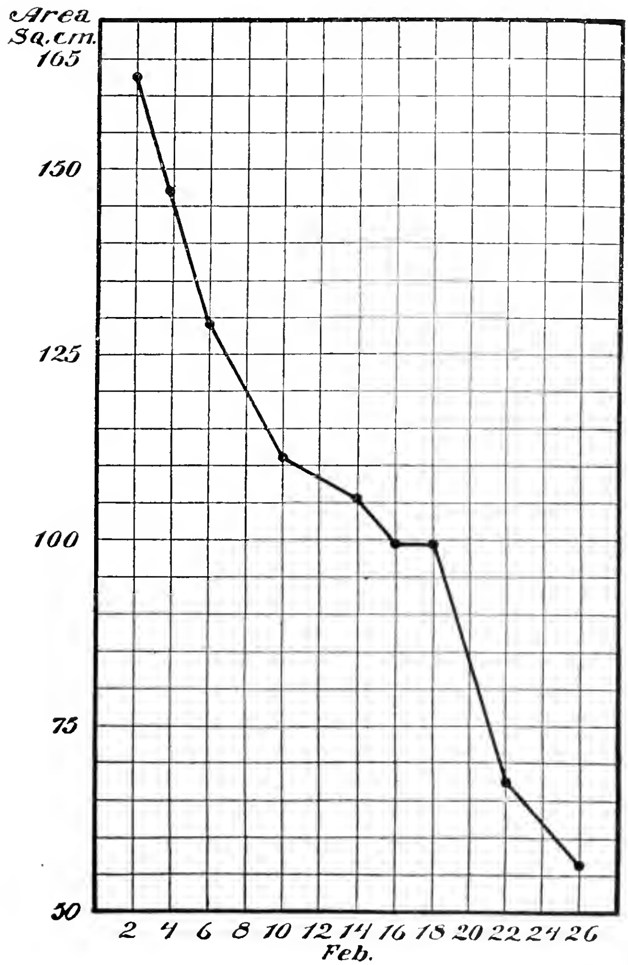
	Feb. 9	12	14	17	21	23	26
S..............................	1.8	2.0	2.0	1.2	0.6	0.3	0
R..............................				0.26	0.15	0.15	0.1



TEXT-FIG. 8.

Experiment 8.—Guinea Pig 4 (Text-fig. 8). Wound of the anterior abdominal wall. The slow rate of cicatrization from Feb. 16 to 21 was due to the period preceding the beginning of contraction.

	Feb. 16	21	23	26	28	Mar. 3	6
S..............................	7.5	5.8	4.6	3.0	2.4	0.8	0.5
R..............................		0.34	0.55	0.36	0.56	0.3	0.4



TEXT-FIG. 9.

Experiment 9.—Patient 360, age 21 years (Text-fig. 9). Wound of the abdominal wall. The horizontal part of the curve from Feb. 16 to 18 represents a period of infection. As soon as the wound was sterilized chemically, the curve descended abruptly.

	Feb. 2	6	10	14	16	18	22	26
S....................	162.5	129.4	111.0	105.5	99.5	99.5	67.5	57.0
R....................		8.25	4.6	1.3	3.0	0	8.0	2.6

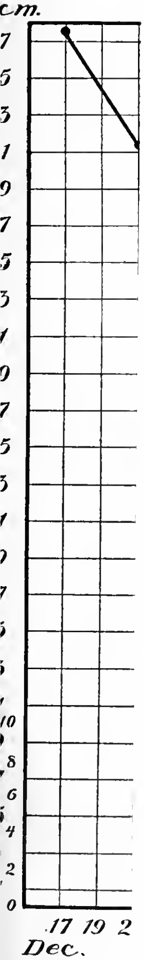
The curves representing the progress of cicatrization in these experiments assume a geometric appearance. It seemed probable, therefore, that the relation between the size of a wound and the rate of repair may be expressed mathematically.[3]

The regularity of cicatrization depends in a large measure on the bacteriologic condition of the wound. The more aseptic the wound, the more regular is the curve of cicatrization. In Experiment 1 the wound was aseptic during the greater part of the period of repair. Agar and bouillon, inoculated with the secretions of this wound, remained sterile. In the other experiments the wounds were slightly infected. After a wound was chemically sterilized the rate of cicatrization increased (Text-fig. 9).

When an aseptic or slightly infected wound was infected, the curve of cicatrization became horizontal or inflected upwards, showing that arrest or retrogression of the repair occurred. In the following experiment a wound accompanying a fracture of the humerus had been almost completely sterilized and was cicatrizing normally, when a slight infection occurred. Cicatrization stopped and the wound enlarged (Text-fig. 10).

Some of the sterile wounds became infected while under observation. As soon as bacteria appeared the process of repair was retarded

[3] du Noüy, P., *J. Exp. Med.*, 1916, xxiv, 451.



Experiment 1

1 Jan. 14 the

TEXT-FIG. 10.

Experiment 10.—Patient 266, age 33 years (Text-fig. 10). Wound and fracture of the left arm. The wound was slightly infected from Dec. 17 to Jan. 14. On Jan. 14 the infection caused the curve to become irregular.

	Dec. 17	21	25	29	Jan. 2	6	10	14	18	22	26	30
S.....	47.3	41.3	35.4	27.3	19.4	15.4	11.7	8.2	9.2	9.6	8.3	7.6
R.....		1.5	1.5	2.0	1.97	1.0	0.92	0.87	0.2	0.1	0.3	0.1

and sometimes stopped. However, infection, when it was of short duration, had no marked disturbing influence on the curve representing the cicatrization of the wound. As soon as normal conditions were reestablished, the progress of cicatrization was hastened (Text-fig. 9). After a period of inactivity an automatic mechanism seemed to accelerate the healing process. This explains why short periods of infection did not produce marked irregularities in the curve of cicatrization. Again, a period of greater activity was generally followed by a period of slow repair which reestablished the regularity of the curve.

In all the experiments cicatrization was more rapid at the beginning than at the end of the period of repair. At the beginning of the process of contraction, the curve inclined rapidly downwards (Text-fig. 7), then the inclination diminished and finally the curve became almost horizontal. The greater activity of the beginning of the granulating period has already been observed. It was generally attributed to the aging of the wound. In previous experiments[1] we found that during the period of contraction, a wound 60 to 70 mm. wide diminished from 9 to 10 mm. in 24 hours. When it was not more than 20 mm. wide the velocity of cicatrization decreased markedly and became very slight when the edges of the wound were located at a distance of only 10 to 12 mm. from each other. In recent experiments the variations of the size of a wound and not only of one of its dimensions were examined. The absolute value of the rate of cicatrization, that is, the surface lost by the wound in 24 hours, depended mainly on the size of the wound. Wounds of from 120 to 150 sq. cm. decreased from 4 to 7 sq. cm. in 24 hours, while the daily diminution of wounds from 10 to 20 sq. cm. was but 1 to 2 sq. cm. In Experiment 1 the wound followed the same law. When its area was 16.2 sq. cm., it diminished at a speed of 1.37 sq. cm. When it attained 1 sq. cm. its velocity became 0.3 sq. cm. It was therefore evident that the rate of repair was proportional to the size of the wound.

The rate of cicatrization, however, was not exactly proportional to the size. For example, in Experiment 1 the area which on Dec. 17 was 16.2 sq. cm. became 1 sq. cm. on Jan. 6. If the equation

$$\frac{S}{S'} = \frac{R}{R'}$$

represented the rate of cicatrization, the rate R' would be expressed thus:

$$R' = \frac{RS'}{S}$$

and its value in Experiment 1 would be $R' = 0.08$. However, it was found that the rate of cicatrization when the size of the wound was 1 sq. cm. decreased only to 0.3 instead of 0.08, and that the complete healing occurred at the end of 6 days. Similar results having been obtained in other experiments it may be concluded that the rate of cicatrization diminishes at the same time as the size but less rapidly. On the other hand, comparison of the figures representing the rate and the area shows that the value of the rate in relation to the size augmented progressively. In Experiment 1 the velocity of cicatrization was successively $\frac{1}{36}$, $\frac{1}{11}$, $\frac{1}{10}$, $\frac{1}{11}$, $\frac{1}{8}$, $\frac{1}{7}$, $\frac{1}{3}$, $\frac{1}{5}$, and $\frac{1}{2}$ of the area. It was found also that the value of the rate in relation to the surface was always greater in a small than in a large wound. If it were otherwise cicatrization would be extremely slow and wounds would not heal.

As stated above, the diminution of the velocity of cicatrization as healing progresses has been generally attributed to the aging of the wound. In reality the phenomenon depends on the reduction of the size of the wound. Former experiments have shown that during a similar period large wounds cicatrized with greater velocity than small ones. In recent experiments made on men and on guinea pigs the rates of cicatrization of wounds of equal area and of different age were compared. Table I shows that wounds of an area of 2 sq. cm. cicatrized completely in from 8 to 10 days and that no relation existed between their age and the velocity of cicatrization.

TABLE I.

Case No.	Age of wound.	Size of wound.	Day of healing.
		sq. cm.	
290	42 days.	2.2	8
354	7 mos.	2.2	8
256	2 "	2	9
330	13 days.	2.1	8
217	2 mos.	2.2	10
221	5 "	2.2	8

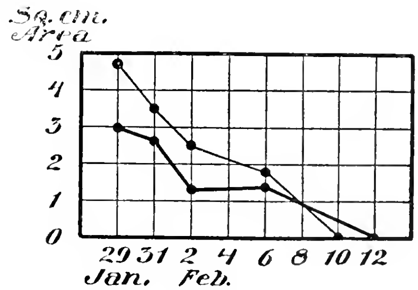
The observations made on guinea pigs gave similar results (Table II).

TABLE II.

Guinea pig No.	Age of wound.	Size of wound.	Day of healing.
	days	*sq. cm.*	
1	7	1.1	8
5	5	1.1	6
2	11	1.1	4
3	11	1.1	5

Since wounds of equal size but of different age cicatrized with variable velocity independently of the age of the wound, the slackening in the speed observed at the end of the period of cicatrization was probably the result of the diminution of the size.

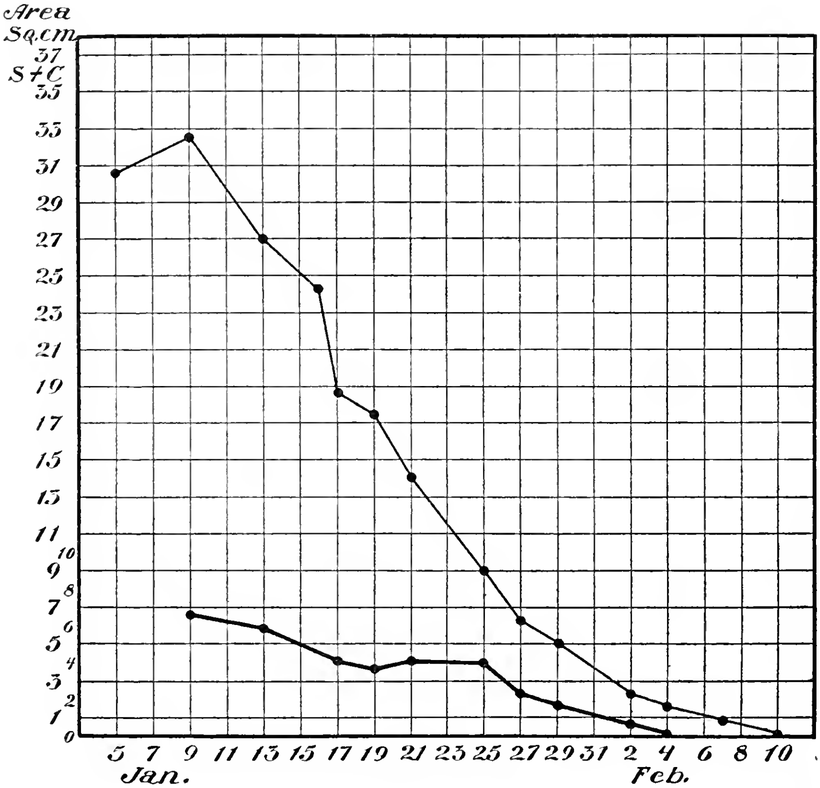
To complete the demonstration of this fact, we have studied wounds of equal age and unequal size. Previous experiments had shown that if two wounds were made at the same time on a dog, the rate of repair of the larger wound was greater than that of the smaller one. So in trapezoidal wounds the reduction of the smaller side was slower than the diminution of the larger side. After a few days, therefore, a trapezoidal wound showed a tendency to become rectangular, circular, or oval. These results were confirmed in the following experiments.



TEXT-FIG. 11.

Experiment 11.—Patient 286, age 30 years (Text-fig. 11). Shell wounds of the clavicular region. The interval between the curves of the larger and smaller wounds became progressively shorter, showing that the rate of repair of the larger wound was greater than that of the smaller one. The larger wound healed before the smaller one.

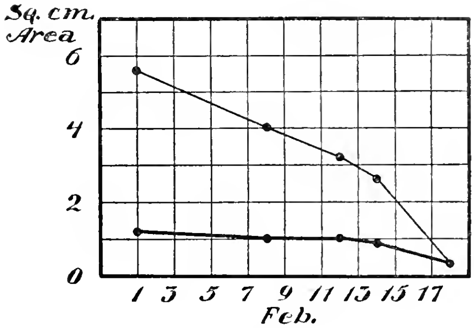
	Area (S) of larger wound.	Area (S') of smaller wound.	$S-S'$	$\frac{S}{S'}$
Jan. 29	4.7	3.0	1.7	1.5
Feb. 6	1.7	1.4	0.3	1 2



TEXT-FIG. 12.

Experiment 12.—Patient 290, age 31 years (Text-fig. 12). Shell wounds with fracture of the radius and cubitus. The curves of both wounds tend to unite.

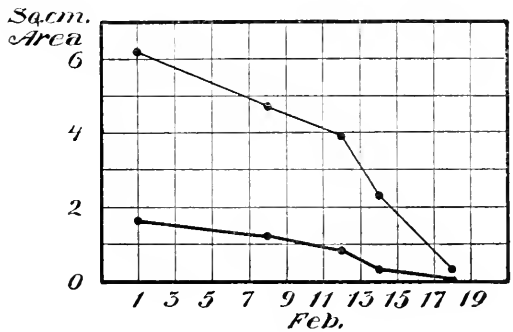
	Area (S) of larger wound.	Area (S') of smaller wound.	$S-S'$	$\frac{S}{S'}$
Jan. 9	32.5	6.7	25.8	4.8
Feb. 2	2.3	0.6	1.7	3.8



TEXT-FIG. 13.

Experiment 13.—Cat 1 (Text-fig. 13). Wounds of the abdominal wall. The curves of both wounds unite at the end of the period of cicatrization.

	Area (S) of larger wound.	Area (S') of smaller wound.	$S-S'$	$\frac{S}{S'}$
Feb. 1	5.6	1.2	4.4	4.6
" 18	0.3	0.3	0	1



TEXT-FIG. 14.

Experiment 14.—Cat 2 (Text-fig. 14). Wounds of the abdominal wall. The curves of both wounds tend to unite.

	Area (S) of larger wound.	Area (S') of smaller wound.	$S-S'$	$\frac{S}{S'}$
Feb. 1	6.2	1.6	4.6	3.8
" 18	0.3	0	0.3	

The graphic expression of these experiments shows that wounds of unequal dimensions have a tendency in the course of cicatrization to become equal in curve. In Experiment 13 a wound of 5.6 sq. cm. and another wound of 1.2 sq. cm. were both reduced after 17 days to

0.3 sq. cm. (Text-fig. 13). It is evident that the lessening in the rate of cicatrization at the end of the period of repair is due to the progressive diminution of the size of the wound and not to its age.

The Value of Contraction and of Epidermization in the Healing of Wounds.

In order to study the relation of contraction and epidermization and the comparative value of both processes, not only the area of the wound had to be known but also that of the cicatrix. The area of the cicatrix was determined by the use of a planimeter on cellophane tracings. The curves expressing the area of the wound and the area of the cicatrix and the wound were separated by an interval which represented in square centimeters the area of the cicatrix at a given time. The value of the epidermization and of the contraction could thus be ascertained every day.

In these experiments errors might arise from lack of distinctness in the outer edge of the cicatrix. It was necessary, therefore, to select wounds whose measurements could be made with sufficient accuracy. The experiments were made on eight wounds on patients of different ages, on a wound made on a guinea pig, and on two wounds made on cats.

Experiment 23.—Cat 1. Wound of the abdominal wall.

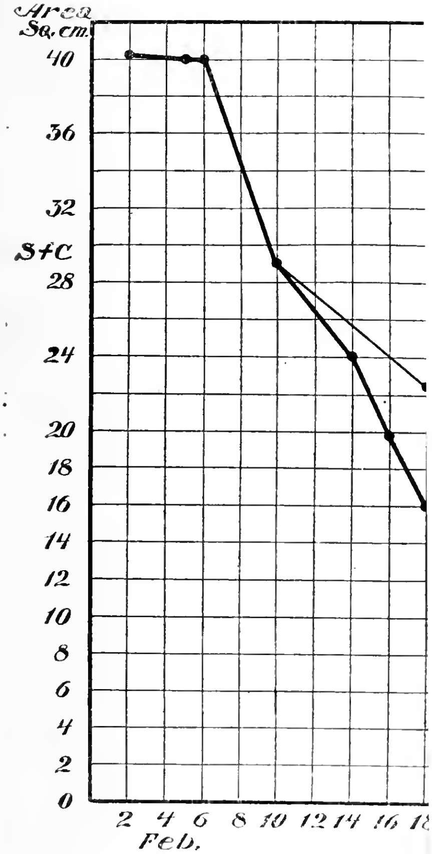
	Feb. 1	8	12	14	18	23
S	5.6	4.0	3.2	2.6	0.35	0
$S + C$	5.6					1.3

Experiment 24.—Cat 2. Wound of the abdominal wall.

	Feb. 11	12	14	18	21	23
S	6.2	3.9	2.3	0.35	0.1	0
$S + C$	6.2					0.5

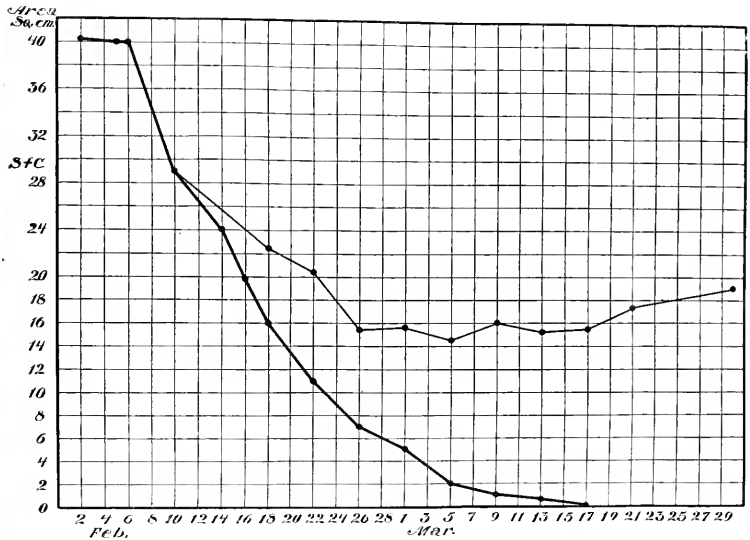
Experiment 25.—Patient 354, age 40 years. Old wound on a stump.

	Jan. 20	22	24	25	27	30	Feb. 1	4	6
S	6.2	4.7	3.5	3.0	2.2	1.3	1.0	0	
$S + C$	42.8				40.0		37.0		40.7



Experiment 15.—Patient 361, ag
The lower curve represents the area
The curve of the wound was horiz
became horizontal on Feb. 26, sh
and not to contraction.

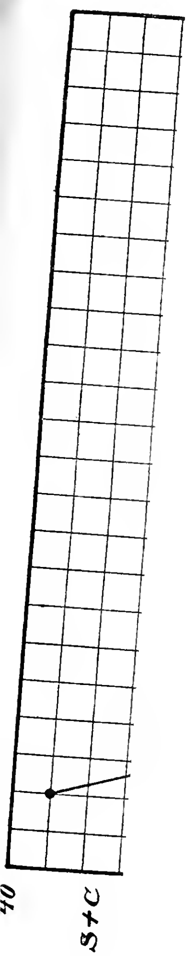
	Feb
S..............	40
S + *C*..........	



TEXT-FIG. 15.

Experiment 15.—Patient 361, age 21 years (Text-fig. 15). Shell wound of the posterior thoracic region. The lower curve represents the area of the wound, and the upper curve the area of both wound and cicatrix. The curve of the wound was horizontal until Feb. 6. Afterwards it descended regularly. The upper curve became horizontal on Feb. 26, showing that the last part of the healing was due to epidermization alone and not to contraction.

	Feb. 2.	6	10	14	16	18	22	26	Mar. 1
S..............	40.2	40.0	29.3	24.0	19.8	16.0	11.0	7.1	5.0
S + *C*..........						22.5	20.5	15.5	15.6



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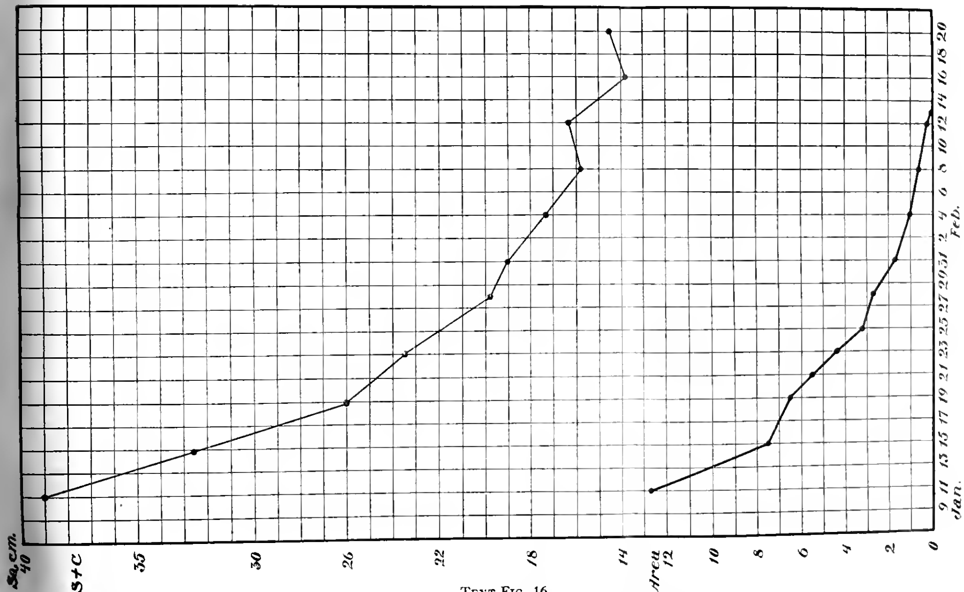
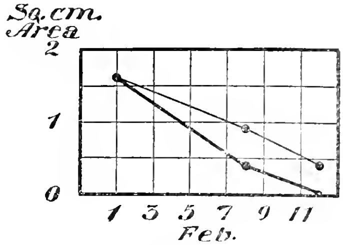


TEXT-FIG. 16.

Experiment 16.—Patient 256, age 21 years (Text-fig. 16). Shell wound of the leg. Both upper and lower curves descend together. This shows that the wound cicatrized at the same time by both processes, contraction and epidermization. After healing was completed, the upper curve ascended, because the cicatrix began to repair.

	Jan. 11	15	19	21	23	25	28	31	Feb. 4	8	12	13
S.....	12.7	7.5	6.5	5.5	4.4	3.2	2.7	1.7	1.0	0.6	0.2	0
S + C	39.0	32.5	26.0		23.5		19.8	19.0	17.4	15.8	16.3	



TEXT-FIG. 17.

Experiment 17.—Guinea Pig 5 (Text-fig. 17). Wound of the abdominal wall. Both curves descend together showing that epidermization and contraction took part in the healing of the wound.

	Feb. 1	8	12
S	1.6	0.4	0
$S + C$	1.6	0.9	0.4

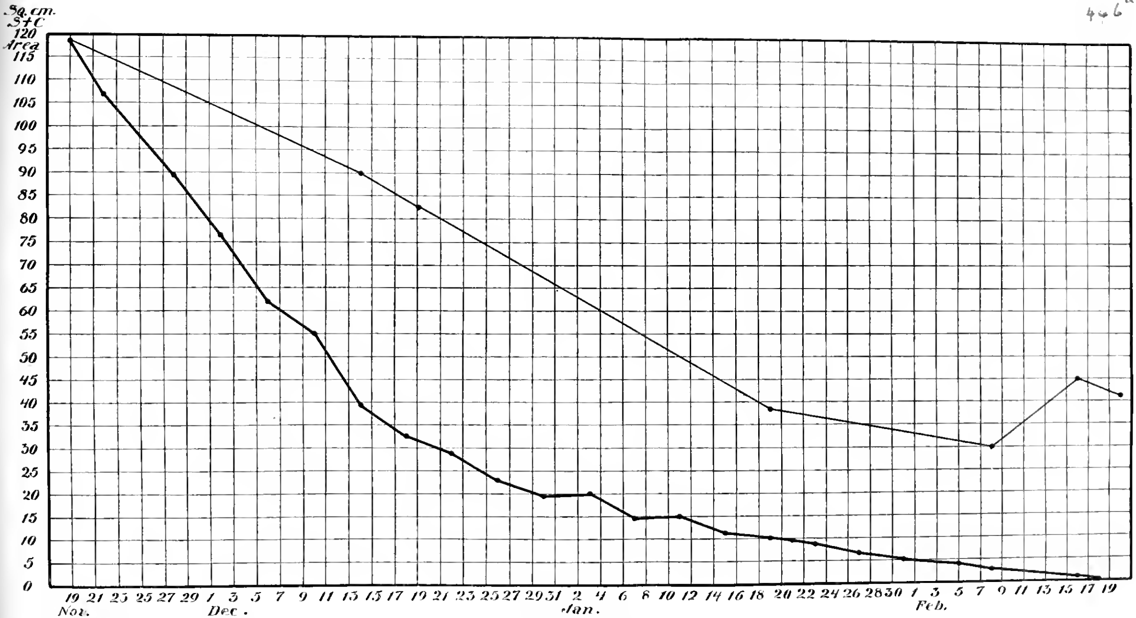
The tracings representing the cicatrization of these wounds demonstrate that contraction was nearly always associated with epidermization. In the experiments previously made on dogs at The Rockefeller Institute, the period of contraction started from 2 to 5 days after the resection of the flap of skin. The beginning of the contraction was preceded by a period during which the tracing had a horizontal direction. Then the curve inflected abruptly downwards and the reparation of the wound began. In Experiment 7 the period of immobility of the wound lasted for 6 days. In Experiment 15 it lasted longer although the wound was covered with granulations. A fragment of shell, remaining in the deep part of the wound, maintained the tissues in a condition of infection which prevented reparation.

After a few days the processes of contraction and epidermization always coexisted. However, there was a period when the wound healed exclusively by contraction. On the curve of Experiment 15 it is seen that during 12 days, contraction alone caused the reparation. Afterwards, epidermization started and the wound cicatrized under the influence of both processes. In most of the wounds observed on human beings contraction and epidermization worked together. Generally contraction continued until complete healing. The curves of the wounds in Experiments 16, 18, and 25 illustrate this phenomenon. However, contraction sometimes stopped before cicatrization was complete. It should be remembered that this process is dependent on contraction of the granulation tissue and on the opposite action of

the tissues surrounding the wound. The resistance of the skin to granulous contraction is very slight in narrow wounds. If the defect is larger, the wound contracts until the elasticity of the surrounding skin prevents a greater reduction of its size. It is also probable that under certain conditions the granulation tissue does not possess the power of contraction. In Experiment 18 contraction did not last until complete healing. The curves representing the cicatrization of the wounds of Experiments 21 and 22 show that during the last days of repair the area of the cicatrix enlarged. In both wounds epidermization assumed almost entirely the effort of repair. As contraction was lacking, epidermization became more rapid and compensated for the dilatation of the cicatrix. It would seem that the processes of contraction and epidermization, although independent in some measure, can each make up for the other's deficiency.

Contraction always activated healing unless retrogression of epidermization occurred simultaneously. The upper curve of Experiment 23 shows a sudden and temporary acceleration of contraction. The lower curve remains parallel to the upper curve. It shows that epidermization had no influence on the rate of contraction. The lower curve indicates a sudden acceleration of the cicatrization, under the influence of a certain substance. During the same period the upper curve remained regular, showing that contraction pursued its normal course while the process of epidermization alone responded to the disturbing action of the dressing. The factors which acted on one of the processes appeared to have been without influence on the other.

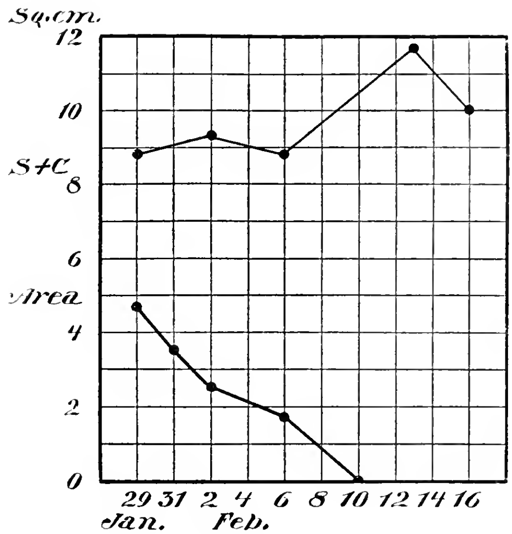
The relative importance of both processes, contraction and epidermization, was valued quantitatively by comparing the area of the cicatrix and that of the wound. The area of the cicatrix represents the result of the epidermization. The difference between the surface of the cicatrix and of the wound gives the value of the contraction. Previous experiments had shown that the cicatrix of a large wound was comparatively smaller than that of a small wound. Two rectangular wounds, one 66 mm. wide and the other 26 mm. wide, were made on the same dog under ether anesthesia. The wound of 66 mm. gave a cicatrix 22 mm. wide, and that of 26 mm. a cicatrix of 13 mm. The width of the cicatrix of the larger wound represented a third of



TEXT-FIG. 18.

Experiment 18.—Patient 263, age 36 years (Text-fig. 18) Wound of the forearm with fracture of the cubitus. Cicatrization is brought about by both processes. As soon as the healing was completed, the cicatrix became larger, as is shown by the upward inflection of the curve.

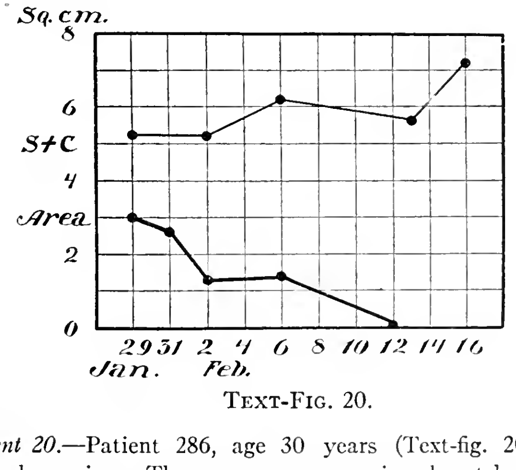
	Nov. 19	28	Dec. 6	14	26	Jan. 7	19	31	Feb. 8	16	20
S..........	118.5	89.6	62.1	39.7	23.0	14.8	10.0	5.2	2.6	0.4	0
S + C.....				90.0			38.5		30.0		41.0



TEXT-FIG. 19.

Experiment 19.—Patient 286, age 30 years (Text-fig. 19). Wound of the upper clavicular region. The upper curve remains horizontal for a while and afterwards goes up. Cicatrization was due entirely to epidermization and not to contraction.

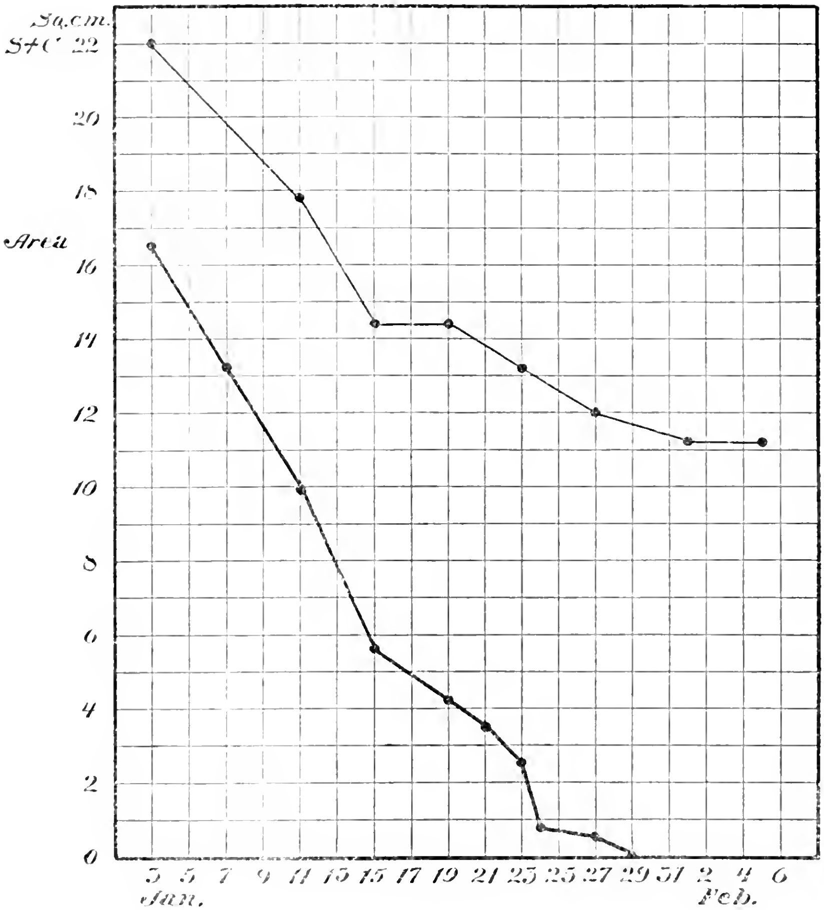
	Jan. 29	31	Feb. 2	6	13
S	4.7	3.5	2.5	1.7	0
S + C	8.8		9.3	8.8	11.7



TEXT-FIG. 20.

Experiment 20.—Patient 286, age 30 years (Text-fig. 20). Wound of the lower clavicular region. The upper curve remains almost horizontal. No contraction. Cicatrization was produced by epidermization.

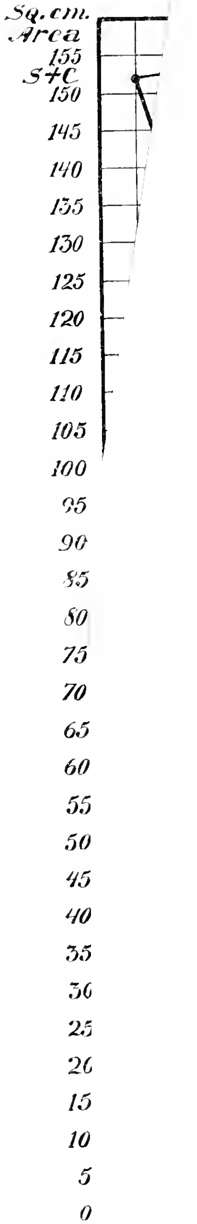
	Jan. 29	31	Feb. 2	6	12	13	16
S	3.0	2.6	1.3	1.4	0		
S + C	5.2		5.2	6.2		5.6	7.2

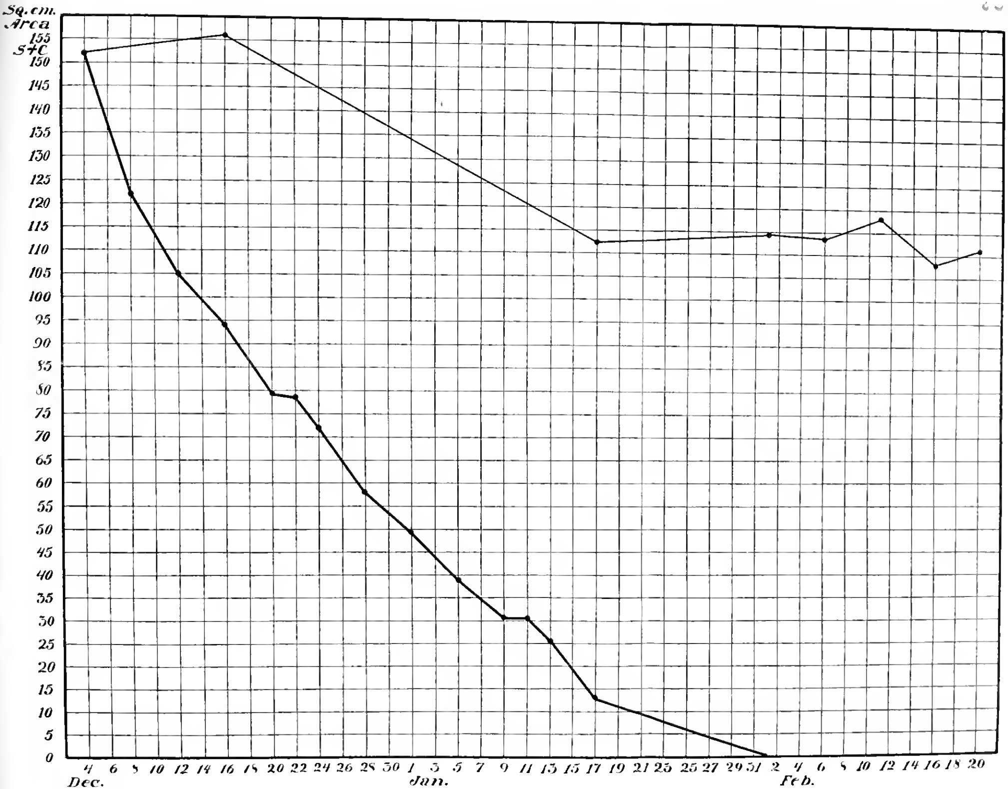


TEXT-FIG. 21.

Experiment 21.—Patient 289, age 29 years (Text-fig. 21). Shell wound of the external part of the leg. On Jan. 15 the downward inflection of the lower curve was the result of the acceleration of contraction. From Jan. 15 to 19, the upper curve remained horizontal while the lower curve went downward. On Jan. 24 the lower curve was irregular while the upper curve was not disturbed, showing that both contraction and epidermization were in some measure independent.

	Jan. 3	7	11	15	19	21	23	24	27	29
S........	16.5	13.2	9.9	5.6	4.2	3.5	2.5	0.8	0.5	0
S + *C*...	22.0		17.8	14.4	14.4		13.2		12.0	


Sq. cm.
Area
155
S+C
150
145
140
135
130
125
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0



TEXT-FIG. 22.

Experiment 22.—Patient 269, age 35 years (Text-fig. 22). Burn of the external surface of the right arm. The large interval between both curves shows that cicatrization was produced chiefly by epidermization. After Jan. 17 contraction ceased altogether.

	Dec. 4	8	16	24	Jan. 5	17	Feb. 5	11
S........	152.0	122.2	94.0	72.0	39.0	12.8	0	0
S + C	152 0		156.0			112.5	113.5	118 0

the width of the wound, and that of the smaller only a half. The cicatrix of a wound of about 1 sq. cm. in size was scarcely smaller than the wound. These facts were confirmed by Experiments 17, 24, 23, and 20. On Guinea Pig 6 three-quarters of the cicatrization was due to the contraction, and the last quarter to epidermization. In Cat 3 both processes acted in similar proportion. In Cat 2 one-twelfth of the cicatrization was due to epidermization. On the contrary, the reparation of the wounds in Experiments 19 and 20 was caused entirely by epidermization. As a rule, contraction played a more important part in healing than epidermization.

Mensuration of the cicatrix at the end of the period of repair showed that contraction stopped as soon as the wound was completely healed. From this time, the size of the cicatrix increased progressively. Thus, the period of contraction was followed by a phase of expansion, as in Text-fig. 15. The wound of Guinea Pig 6, the width of which was 4 mm. at the time of healing, measured 7 mm. 5 days later. The area of the cicatrix of Patient 354 in Experiment 25, which was 37sq. cm. the day of healing, reached 40 sq. cm. 5 days later. The cicatrix of Patient 269 which was 113.5 sq. cm. had an area of 118 sq. cm. 6 days later. Expansion was much less marked in man than in the dog. In animals whose experimental wounds were produced by resection of a large flap of skin, the dilatation of the cicatrix was much larger. It spread slowly for a long time in such a way that the scar might become as large as the original wound.

CONCLUSIONS.

1. A method for measuring the area of a wound not geometric in form is described.

2. The rate of cicatrization of a wound is greater at the beginning than at the end of the period of repair. It depends on the area rather than on the age of the wound. There is a constant relation between the size of a wound and the rate of cicatrization. The larger the wound the greater is the rate of cicatrization. Two wounds of different size have a tendency to become equal.

3. The rate is proportional to the area, but diminishes less rapidly than the area.

4. The process of contraction is the more important factor in the repair of a wound. Epidermization completes the work of contraction. After the wound is healed, the cicatrix as a rule expands.

5. The curve representing the diminution of the size of an aseptic wound while it cicatrizes is regular and geometric.

CICATRIZATION OF WOUNDS.

II. Mathematical Expression of the Curve Representing Cicatrization.

By P. LECOMTE DU NOÜY.

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(Received for publication, May 24, 1916.)

In order to study the process of cicatrization, a technique for measuring accurately the area of wounds was developed. Sterilized cellophane was applied to the wound and the edge was outlined with a wax pencil. This drawing was transferred in ink to an ordinary sheet of paper. Afterwards the area was measured by means of a planimeter, either the Amsler system or some other. A curve was obtained by carrying the area, in square centimeters, in ordinates, and the time, in days, in abscissæ.

In many experiments made by Dr. Carrel the curve representing the cicatrization of aseptic wounds was of regular and geometric appearance. These curves were expressed by a mathematical equation in function of time and area.

After a large number of slightly infected wounds had been studied, a simple extrapolation formula was obtained. Marked deviation from the calculated curve showed generally that infection had set in. By means of the formula the area of the wound after a given time can be foreseen. The formula may be expressed in two equations.

$$(1) \quad \frac{\frac{S - S'}{S}}{t + \sqrt{T}} = i$$

$$(2) \quad S'' = S' [1 - i (t' + \sqrt{T + t'})]$$

S represents the area of the wound at the beginning of the experiment.

S' represents the area of the wound t days later, at the time of the second observation. (We may say 4 days, in order that the area cicatrized, $S-S'$, may be of sufficient size.)

t represents the time elapsed between the first two observations, S and S', in days.

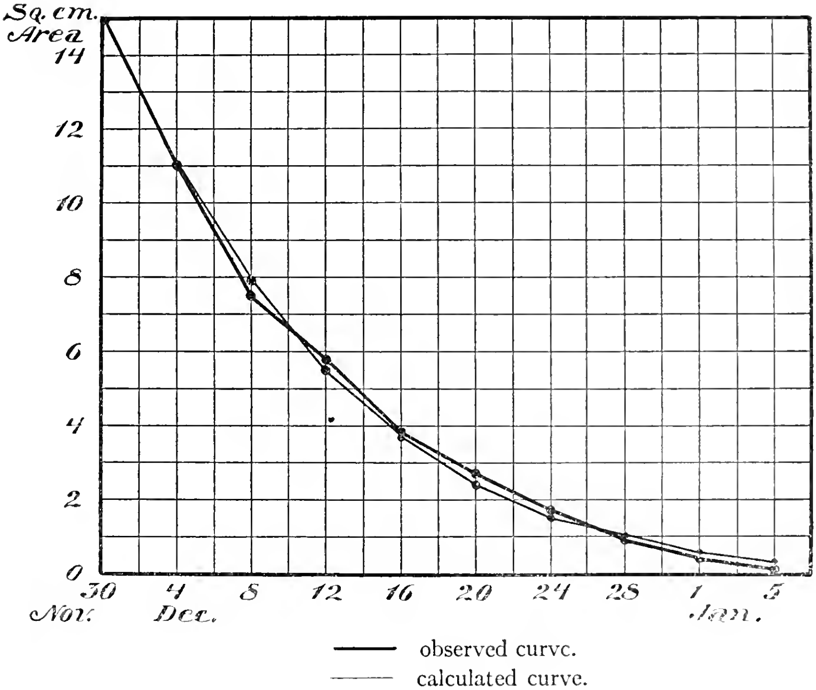
T represents the age of the wound from the time of the first observation S. Therefore in the formula (1) $T = t$, and practically $t = t' = 4$ days.

t' represents the time between the last observation S' and the time of the theoretical surface S'' of the wound.

i is a constant coefficient characterizing each wound.

Thus the first equation tends to establish an index of cicatrization i which is carried into the second in order to calculate the surface of the wound at a given time. It should be noticed that the best approximation is obtained when the time t is the same as the time t'. Therefore, after two observations, 3 or 4 days apart, of a wound which heals aseptically, the area that it should have 4, 10, 20, or 30 days later, and the day on which it should be healed can be computed. Accidents, of course, may retard temporarily the progress of the phenomenon. But generally the time lost is regained by a rapid acceleration as shown in Wounds 4, 6, and 7.

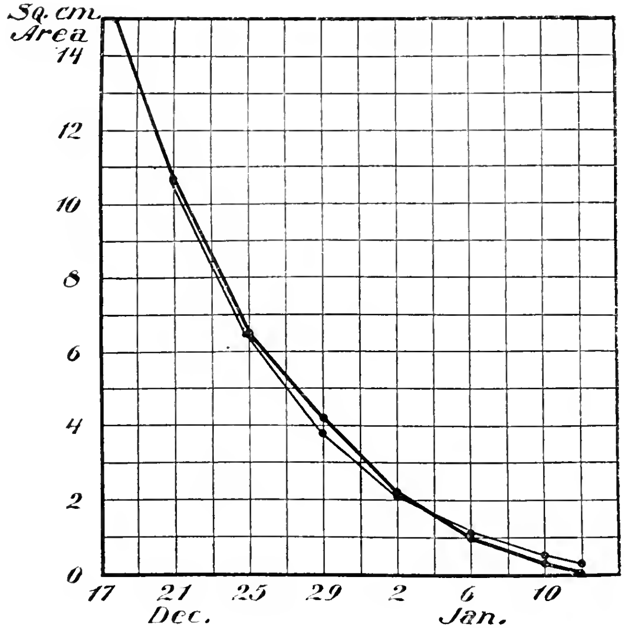
In the following experiments the calculated areas are compared with the observed areas.



TEXT-FIG. 1.

Experiment 1.—Patient 217, age 37 years. Wound of the arm. On Nov. 30, the size was 15.3 sq. cm. Index = 0.041 (Text-fig. 1). Between Dec. 18 and 24, a few bacteria appeared on the films (1 to 3 per microscopic field).

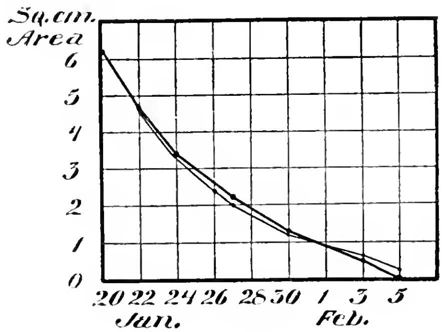
	1915 Dec. 4	8	12	16	20	24	28	1916 Jan. 1	5
Observed area..	11.0	7.5	5.8	3.8	2.7	1.7	0.9	0.4	Cicatrization.
Calculated " ..		7.9	5.5	3.7	2.4	1.55	0.96	0.58	"



TEXT-FIG. 2.

Experiment 2.—Patient 221, age 27 years. On Dec. 17 the area of the wound was 16.2 sq. cm. Index = 0.057 (Text-fig. 2). There was a slight infection between Dec. 27 and 29 (1 microorganism in two microscopic fields).

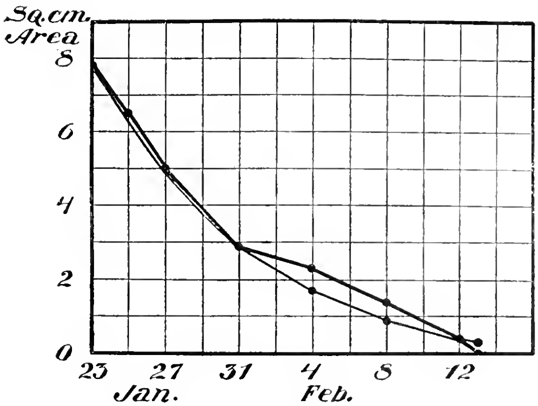
	1915 Dec. 21	25	29	1916 Jan. 2	6	10	12
Observed area.............	10.7	6.5	4.2	2.2	1.0	0.3	Cicatrization.
Calculated "		6.5	3.8	2.1	1.1	0.5	"



TEXT-FIG. 3.

Experiment 3.—Patient 354, age 40 years. On Dec. 20 the area of the wound was 6.2 sq. cm. Index = 0.07 (Text-fig. 3).

	1916 Jan. 22	24	27	30	Feb. 3	5
Observed area....................	4.7	3.4	2.2	1.3	0.5	Cicatrization.
Calculated "		3.4	2.4	1.2	0.54	"

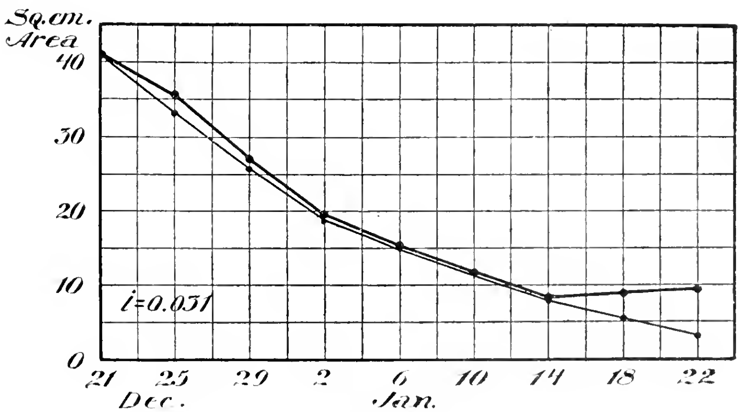


TEXT-FIG. 4.

Experiment 4.—Patient 330, age 31 years. On Jan. 23 the area of the wound was 7.8 sq. cm. Index = 0.059 (Text-fig. 4).

	1916 Jan. 25	27	31	Feb. 4	8	12	13
Observed area.............	6.5	5.0	2.9	2.3	1.4	0.4	Cicatrization.
Calculated "		5.0	2.98	1.7	0.9	0.44	"

Between February 3 and 8 the wound became infected and deviated from the theoretical curve. However, it overtook the latter on February 12 after it was again sterilized. This shows that acceleration of the cicatrization has occurred, which always happened in the cases which we studied.

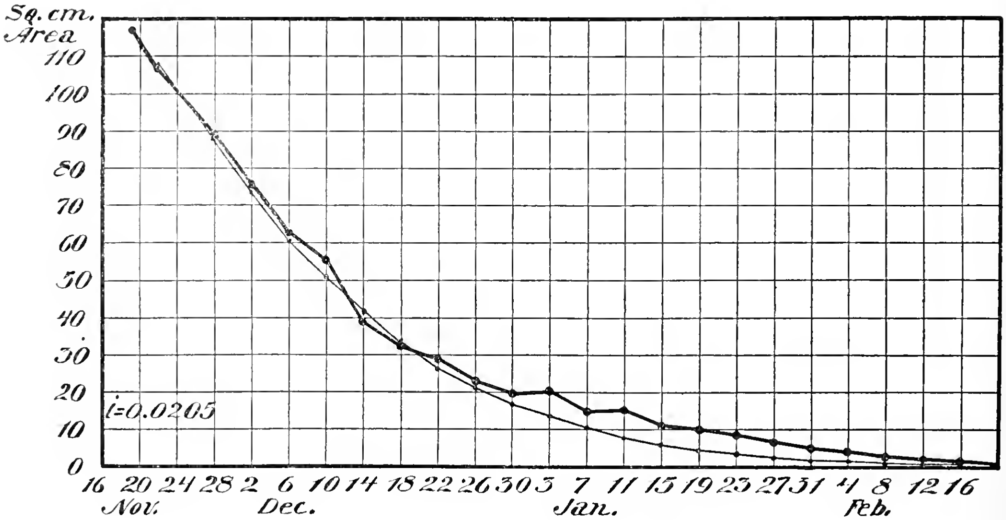


TEXT-FIG. 5.

Experiment 5.—Patient 266, age 33 years. On Dec. 25 the area of the wound was 35.4 sq. cm. Index = 0.031 (Text-fig. 5). On Jan. 14 the wound became infected and began to increase in size. It deviated from the theoretical curve and did not meet it again.

	1915 Dec. 29	1916 Jan. 2	6	10	14	18
Observed area..............................	27.3	19.4	15.4	11.7	8.2	9.0
Calculated "		19.1	15.6	11.3	8.0	5.6

The following experiment was similar, but the observed curve coincided with the calculated curve before healing. The date of healing could be predicted 90 days in advance.

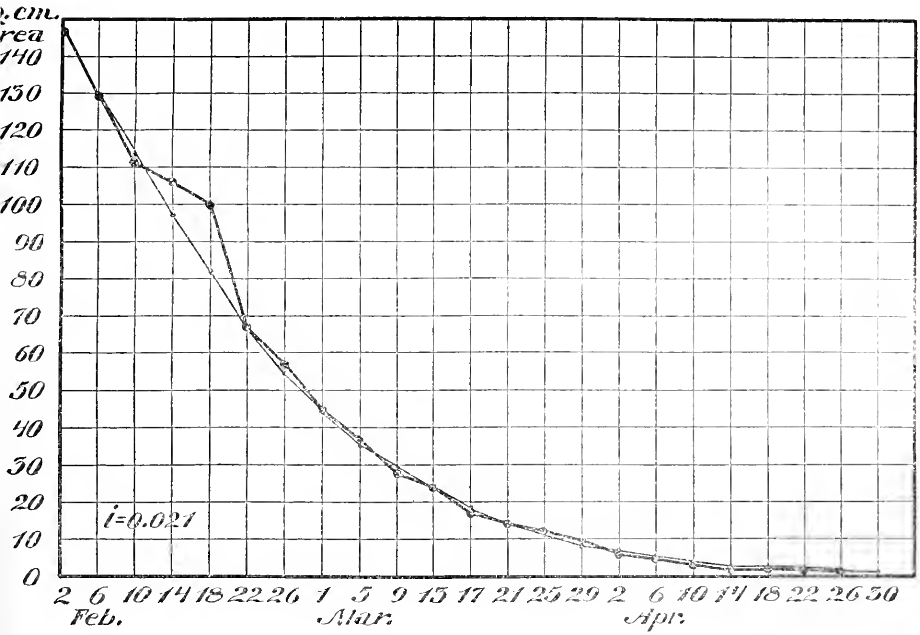


TEXT-FIG. 6.

Experiment 6.—Patient 263, age 36 years. On Nov. 19 the area of the wound was 118.5 sq. cm. Index = 0.0205 (Text-fig. 6). The fluctuations of the curve were due to infection. The observed curve has, as always, a tendency to regain time lost in order to rejoin the calculated curve (from Dec. 10 to 14, then from Jan. 27 to Feb. 18).

	1915 Nov. 22	28	Dec. 2	6	10	14	18	22	26	30
Observed area......	107.0	89.6	76.0	62.1	55.1	39.7	32.5	29.1	23.0	19.5
Calculated "		88.0	74.2	61.8	51.0	41.6	33.6	26.9	21.3	16.8

	1916 Jan. 3	7	11	15	19	23	27	31	Feb. 4	8	12	16	18
Observed area.....	20.0	14.8	15.0	11.0	10.0	8.5	6.5	5.2	4.3	2.6	1.7	0.4	Cicatrization.
Calculated area..	13.1	10.1	7.8	5.9	4.5	3.4	2.5	1.9	1.4	1.0	0.74	0.5	"



TEXT-FIG. 7.

Experiment 7.—Patient 360, age 22 years. Index = 0.021 (Text-fig. 7). The deviation at the beginning (Feb. 18) was due to infection. After sterilization it overtook the calculated curve.

	1916 Feb. 6	10	14	18	22	26	Mar. 1	5
Observed area...............	129.4	111.0	105.5	99.5	67.5	57.0	45.0	37.0
Calculated "		113.0	96.8	81.6	67.9	55.9	45.5	36.6

	Mar. 9	13	17	21	25	29	Apr. 2
Observed area......................	27.5	23.9	17.6	14.0	11.8	9.2	5.9
Calculated "	29.2	23.1	18.1	14.0	10.8	8.26	6.27

	Apr. 6	10	14	18	22	26	30	May 4	6
Observed area.........	4.1	3.0	1.7	1.8	1.0	0.65	0.7		Cicatrization.
Calculated "	4.7	3.5	2.6	1.9	1.4	1.0	0.7	0.5	"

As shown by the above experiments, 0 of the formula, that is, the theoretical healing, corresponds to an area of about 0.4 sq. cm. This is required by the fact that if the calculation should be continued, 0 would be reduced to infinity. Experience has proved that the calculated number 0.4 nearly always represents complete healing of the wound.

The value of the index varies between 0.02 and 0.08. It depends on the age of the patient and the size of the wound, and is much larger when the wound is smaller and the subject younger.

In conclusion, it may be stated, that, under given conditions of asepsis and dressing, the area cicatrized in a day is directly proportional to the size of the wound, to the square root of its age, and to the relation between the rate of cicatrization and the square root of the age of the wound at the time of observation. The relation between the rate expressed in function of the total area, and the square root of the age of the wound is represented by the cicatrization index i, which acts in Formula 2 as a constant characterizing each wound.

Mechanism of the Formula.

The study of a wound in the process of healing shows that the age of the wound seems to have no action whatever on the rate of cicatrization. On the other hand, since all wounds under a certain size cicatrize at about equal rate, the part of the constant i is no longer clear, as this index is not the same for every wound. If the age of the wound is of no consequence, it should be suppressed, and the index i, which has after all the dimensions of an acceleration $\frac{V}{T}$, should tend towards the same value for all wounds when they are nearly healed. Therefore i would not be a constant characterizing every wound. How then can this index be of value in the formula as a constant? And what part does the square root of the age of the wound play?

The explanation is simple. The most important factor of the rapidity with which a wound cicatrizes is its size. Consequently when a large wound cicatrizes and grows old, the measure of the progress of cicatrization is its age. Time is one of the factors of its dimensions. Then, if age itself does not influence the rate of cicatrization, the size plays an important part. Therefore a quantity representing the age, a known quantity, represents also in some measure the size of the wound, an unknown quantity. Thus a factor inactive in it-

self, such as age, plays the part of the principal factor, that is size. But it should be understood that the area is expressed in time.

Hence the part of the index i as a constant is explained. In order to express what really occurs, i should increase in a manner inversely proportional to the size, which conforms with the formula:

$$\frac{\frac{S-S'}{S}}{t}$$

but then, the unnecessary element T must be suppressed. Practically' the coefficient i is constant, but we arbitrarily maintain the quantity T in the formula. The result is the same, because, like i, T increases in inverse proportion to the area of the wound. So the progress of cicatrization can be indirectly expressed. This procedure was used, since time was the only factor at our disposal for the calculation of the entire curve of the wound after the first two observations had been made.

Details of the Calculation.

We see from Formula 2 that in order to continue the calculation and obtain successively the area S''' at the end of t'' days, S'''' at the end of t''' days, S_n at the end of t_m days, and so on, the last calculated area is brought into the formula in order to obtain the next area. Then the following calculations are made:

$$\begin{array}{ll} S'' = S' - [S' \times i \times (t' + \sqrt{T+t'})] = S'' & (\text{here: } T = t) \\ S''' = S'' - [S'' \times i \times (t'' + \sqrt{T+t''})] = S''' & (\text{"} \quad T = t + t') \\ S'''' = S''' - [S''' \times i \times (t''' + \sqrt{T+t'''})] = S'''' & (\text{"} \quad T = t + t' + t'') \\ \cdots\cdots\cdots\cdots & \cdots\cdots \\ S_n = S_{n-1} - [S_{n-1} \times i \times (t_m + \sqrt{T + t_m})], & (T = t + t' + t'' + \cdots + t_{m-1}) \end{array}$$

Let us take for example a sterile wound of 12 sq. cm. 4 days later it measures 9 sq. cm.

(1) Calculation of the index i:

$$S = 12 \qquad S' = 9 \qquad t = 4 \qquad T = 4 \qquad i = \frac{\frac{12-9}{12}}{4 + \sqrt{4}} = 0.0416$$

(2) Calculation of the surface:

$$(a)\ 4\ \text{days later}\ \begin{cases} S'' = S' - [S' \times i \times (t' + \sqrt{T + t'})] \\ = x = 9 - [9 \times 0.0416 \times (4 + \sqrt{4 + 4})] = 6.45\ \text{sq. cm.} \end{cases}$$

$$(b)\ 3\ \text{days later}\ \begin{cases} S''' = S'' - [S'' \times i \times (t'' + \sqrt{T + t''})] \\ = 6.45 - [6.45 \times 0.0416 \times (3 + \sqrt{8 + 3})] = 4.84\ \text{sq. cm.} \end{cases}$$

and so on.

11 days after the first observation, the area will be 4.8 sq. cm., if the wound has remained aseptic.

SUMMARY.

The cicatrization of sterile wounds may be studied in the same way as an ordinary physicochemical phenomenon. It is possible, therefore, to express the law of cicatrization by a mathematical equation as soon as an accurate measure of the wound can be obtained. By means of the equation, a curve is obtained which represents the theoretical evolution of the cicatrization of a wound. This curve, being an expression of what should happen on a normal wound, healing aseptically, on a normal man, is a daily point of comparison to what appears actually on the observed wound, and allows one to study accurately the fluctuations of cicatrization on a given individual, and the action of different dressings and antiseptic substances.

CICATRIZATION OF WOUNDS.

III. The Relation between the Age of the Patient, the Area of the Wound, and the Index of Cicatrization.

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(Received for publication, June 20, 1916.)

The observation of many wounds in the process of cicatrization, on patients of different ages, has shown that there is a proportional relation between the age of the patient, the area of the wound, and the index calculated by means of the formula

$$(1)\qquad \frac{\dfrac{S - S'}{S}}{t + \sqrt{T}} = i$$

In other words, one of these quantities may be considered a continuous function of the other two.

A chart has been plotted, and from the curves thus obtained, the intermediate points may be computed without calculation (Text-fig. 1). The index, the only unknown quantity, may thus be obtained directly. It then becomes unnecessary to take two measurements of the wound, 4 days apart, S and S', and one no longer needs to calculate the index i by Formula 1. On the other hand, it is evident that the index is purely theoretical, or rather an average index, which expresses normal cicatrization on a normal individual, and that marked differences may be observed between the index of a given individual, according to his general condition, and the index of the average individual of the same age.

In the course of many experiments it was found that, as a rule, the average, or normal index, was practically the same as the calculated index. Table I shows some of the figures used in making the curves.

TABLE I.

Area.		Index.	Age.	No. of patient.	Similar observations (No. of patient).
sq. cm.			yrs.		
130—	......				
	129	0.0236	22	360	
125 -	......				
120—	......				
	118	0.021	36	263	
115 -	......				
110—	......				
105 -	......	0.022	35	269	
100—	101	0.0264	21	327	
95 -	......				
90—	......				
85 -	.....				
80—	......				
75 -	......				
70—	......	0.0225	27	366	
65 -	......				
	64	0.020	32	318	
60—	......				
55 -	......				
50—	......				
	46	0.0223	38	408	
45 -	......				
40—	......	0.0445	20	361	
35 -	......	0.031	33	266	
30—	......	0.05	21	Ja. (75)	444
25 -	......	0.03	38	408	383
20—	......				
	16	0.057	27	221	289, 300, 336
15 -	......	0.046	37	217	408, 403, 450
	12	0.068	22	256	
10—	......	0.065	29	289	479, 415, 366 (2 experiments).
	7	0.060	31	330	
5 -	......	0.070	39	354	
	2	0.070	30	286	409 (2 experiments).
0—	......				

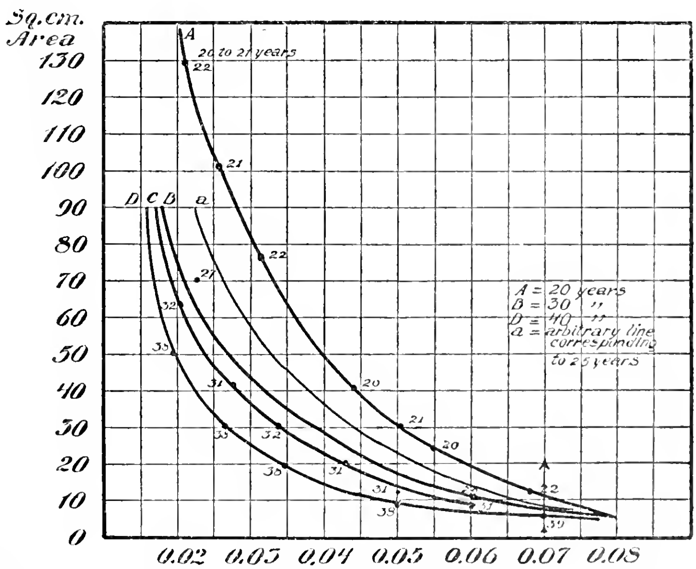
The figures show that the index is generally small for the larger wounds (about 0 020), that it increases for the smaller wounds, and that, the area of the wounds being the same, young individuals have the largest index. Since the index indicates the activity of cicatrization, the formula may be expressed:

$$\frac{ds}{dt} = f\,(A, i)$$

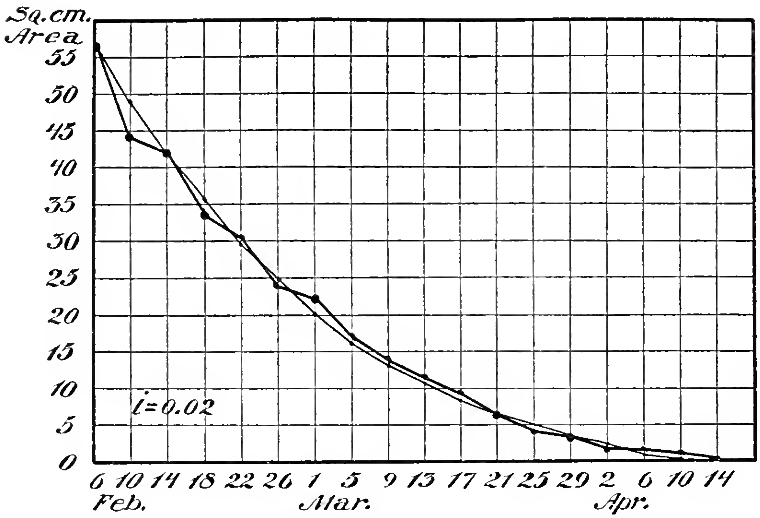
in which A is the age of the patient; S, the area of the wound; i, the index. The curves representing the ages are of the general form: $S^a \times i = K$ (K being a constant).

Text-fig. 1 has been plotted from thirty-five observations of wounds, taken at the beginning of sterilization.

The curves in Text-figs. 2 to 9 have been calculated by means of the index given in Text-fig. 1.



TEXT-FIG. 1. Composite curve for obtaining index directly.



—— observed curve.

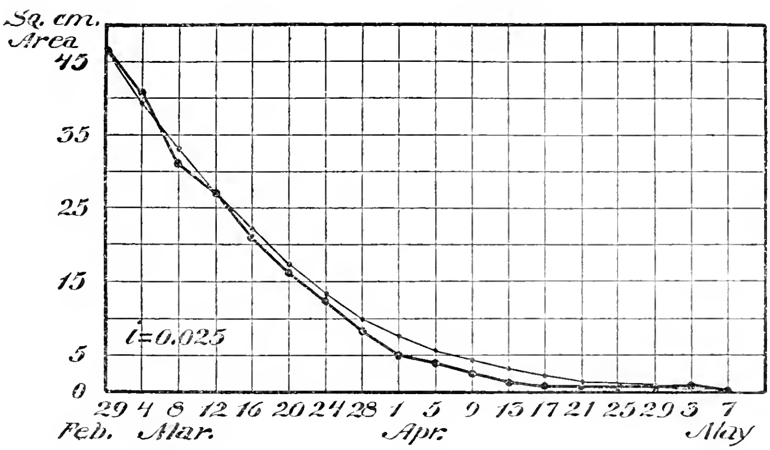
—— calculated curve.

TEXT-FIG. 2.

Experiment 23.—Patient 318, age 32 years (Text-fig. 2). Index = 0.02.

	1916 Feb. 6	10	14	18	22	26	Mar. 1	5	9	13	17
Observed area.	56.6	44.3	42.0	33.6	30.2	24.2	22.1	17.0	13.6	11.0	9.2
Calculated "		49.1	42.0	35.4	29.7	24.9	20.1	16.4	13.2	10.5	8.3

	Mar. 21	25	29	Apr. 2	6	10	14
Observed area.............	6.5	4.2	3.1	1.8	1.15	0.8	Cicatrization.
Calculated "	6.6	4.3	3.1	1.8	0.70	0.2	"

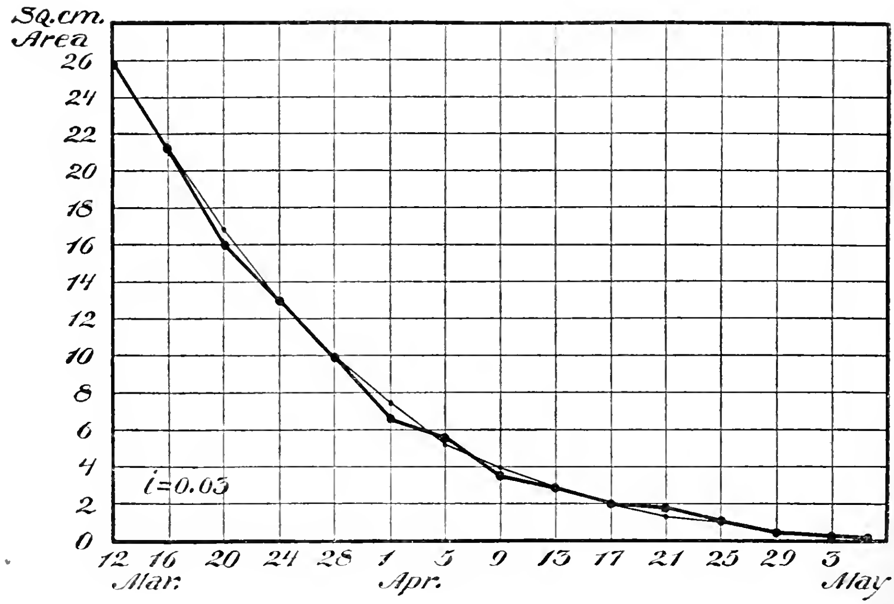


TEXT-FIG. 3.

Experiment 27.—Patient 408, age 38 years (Text-fig. 3). Wound of the thigh. Index = 0.025.

	1916 Feb. 29	Mar. 4	8	12	16	20	24	28	Apr. 1	5
Observed area......	46.7	40.5	31.1	27.0	21.1	16.0	12.5	8.3	5.0	4.0
Calculated "		39.7	32.9	26.8	21.4	16.9	13.1	10.0	7.5	5.6

	Apr. 9	13	17	21	25	29	May 3	7
Observed area.......	2.3	1.4	1.0	1.0	0.9	0.5	0.7	Cicatrization.
Calculated "	4.2	3.1	2.2	1.6	1.1	0.8	0.6	"

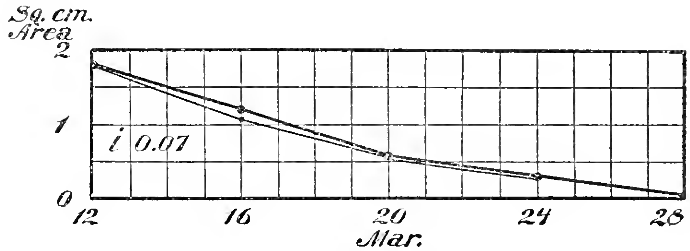


TEXT-FIG. 4.

Experiment 28.—Patient 408, age 38 years (Text-fig. 4). Wound of the arm. Index = 0.03.

	1916 Mar. 12	16	20	24	28	Apr. 1	5	9	13	17	21
Observed area.	25.8	21.1	16.0	13.0	9.9	6.5	5.6	3.6	2.7	2.0	1.7
Calculated "		21.1	16.8	13.0	9.9	7.4	5.4	3.9	2.7	1.9	1.3

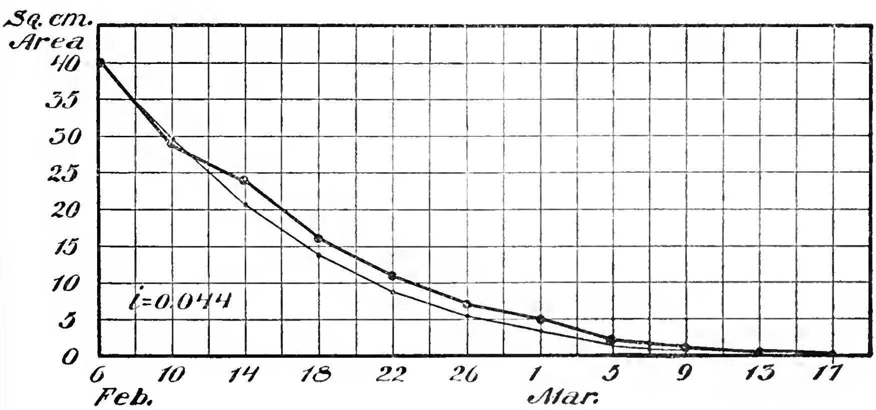
	Apr. 25	29	May 3	7
Observed area..............................	0.9	0.5	0.3	Cicatrization.
Calculated "	0.88	0.59	0.4	"



TEXT-FIG. 5.

Experiment 29.—Patient 408, age 38 years (Text-fig. 5). Wound of the arm. Index = 0.07.

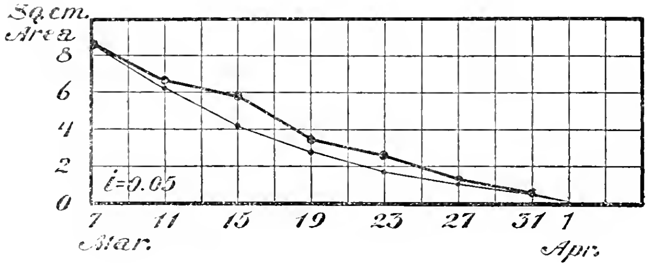
	1916 Mar. 12	16	20	24	28
Observed area..........................	1.8	1.2	0.6	0.3	Cicatrization.
Calculated "		1.05	0.55	0.27	"



TEXT-FIG. 6.

Experiment 30.—Patient 361, age 20 years (Text-fig. 6). Wound of the back. Index = 0.044. Wound infected.

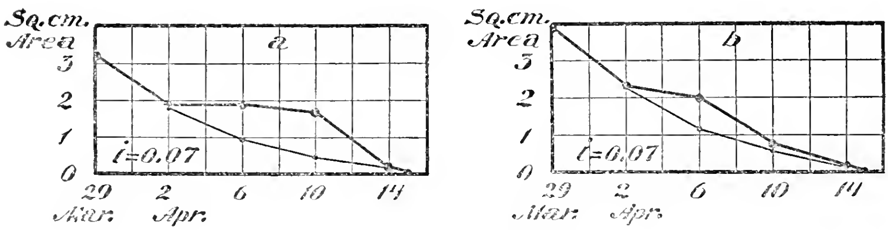
	1916 Feb. 6	10	14	18	22	26	Mar. 1	5	9	13	17
Observed area.	40.0	29.3	24.0	16.0	11.0	7.1	5.0	2.0	1.0	0.65	Cicatrization.
Calculated "		29.4	20.5	13.7	8.9	5.5	3.3	1.8	1.1	0.64	"



TEXT-FIG. 7.

Experiment 31.—Patient 403, age 40 years (Text-fig. 7). Index = 0.05.

	1916 Mar. 7	11	15	19	23	27	31	Apr. 1
Observed area.......	8.7	6.6	5.6	3.4	2.5	1.2	0.5	Cicatrization.
Calculated "		6.1	4.1	2.8	1.7	1.0	0.5	"



TEXT-FIG. 8.

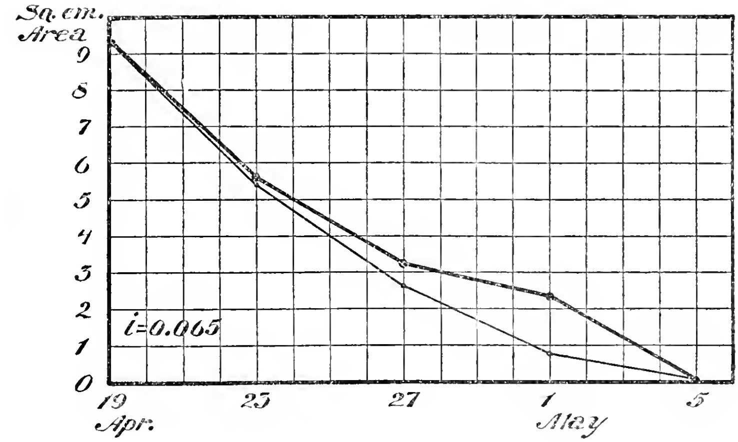
Experiments 32 and 33.—Patient 366, age 27 years (Text-fig. 8). Index = 0.07

Wound A.

	1916 Mar. 29	Apr. 2	6	10	14	15
Observed area....................	3.2	1.9	1.9	1.7	0.2	Cicatrization.
Calculated "		1.85	0.96	0.46	0.21	"

Wound B.

	1916 Mar. 29	Apr. 2	6	10	14	15
Observed area....................	3.9	2.3	2.0	0.75	0.2	Cicatrization.
Calculated "		2.25	1.2	0.56	0.25	"



TEXT-FIG. 9.

Experiment 34.—Patient 415, age 31 years (Text-fig. 9). Index = 0.065.

	1916 Apr. 19	23	27	May 1	5
Observed area	9.3	5.6	3.2	2.3	Cicatrization.
Calculated "		5.4	2.6	0.72	"

SUMMARY.

The first article of this series showed that it was possible to express mathematically the phenomenon of cicatrization. The principal point consisted in determining by means of an equation, a constant, or index, characterizing each wound. The calculation had to be made for each patient for each wound, and required two observations, 4 days apart.

The index having proved to be a continuous function of the size of the wound and of the age of the patient, of the form

$$S^a \times i = K$$

where S is the area, i the index, a a decimal exponent, and K a constant, it was then possible to draw a chart by means of which this index i could be obtained without calculation.

The advantage of the new way of determining the index is, above all, that this index is a general, average, normal index, and no longer an individual index. Hence, the differences between the observed

rate of cicatrization of man and the normal rate may give some indication of the general state of the patient. Another advantage is that the determination of the index is no longer controlled by the temporary accidents which may happen between the two observations of S and S'.

THE PROPHYLAXIS OF WEIL'S DISEASE (SPIROCHÆTOSIS ICTEROHÆMORRHAGICA).

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The prevention of Weil's disease can be undertaken on the basis of the excretion of the pathogenic cause in the urine and feces of patients, and on the routes of invasion of the spirochetes into the human body. But prophylaxis by active immunization seems to offer the surest method.

Prophylaxis by Active Immunization.

Before proceeding to the active immunization of the human body we made preliminary experiments on guinea pigs. The materia, to be inoculated (vaccine) was made in the following manner: To the liver emulsion or the pure culture (in Noguchi's culture medium as modified by Inada and Ido), which contained from 10 to 15 spirochetes in a single field,[1] carbolic acid was added in the proportion of 0.5 per cent, after which the mixture was left for 1 week in the ice box. The clear supernatant fluid was employed for injection into the peritoneal cavity of the guinea pigs three times at intervals of 7 to 9 days. The quantity introduced at the first injection was 2 to 3 cc., at the second 2 to 4 cc., and at the third 2 to 4 cc. The immediate effect of the injection was sometimes to cause feeble convulsions of the body, which disappeared completely in half an hour. The preservative may be responsible for this effect. The temperature often rose to 38–40°C. and continued there for 1 to 2 days. In some instances the guinea pigs, as a result of repeated inoculations, lost weight and appetite, and died. From 9 to 18 days after the last inoculation, 2 to 3 cc. of a liver emulsion or of the pure culture con-

[1] $\frac{1}{12}$ oil immersion, oc. 3 (Leitz), under dark-field illumination.

TABLE I.

Animal No.	Date and quantity of serum inoculated.			Injection of spirochetes.		Result.	Day of disease of control animals.	Result of Pfeiffer's test with blood serum of experimental animals.
	I	II	III	Date.	Quantity.			
1	May 26 2 cc.	June 1 2 cc.	June 9 2 cc.	June 26	Liver emulsion 2 cc.	Died on same day.		
2	"	"	"	" 24	"	Condition good until 23rd day. Killed.	Died of icterus on 5th day.	Positive.
3	May 1 2 cc.	May 24 2 cc.	May 30 2 cc.	" 17	"	Died on following day. Autopsy findings negative.		
4	June 26 2 cc.	July 4 2 cc.	July 10 4 cc.	July 16	"	Died on 8th day. No icterus. Autopsy findings negative.		
5	July 4 2 cc.	July 10 4 cc.	July 16 4 cc.	" 26	Pure culture 2 cc.	Condition good until 19th day. Killed.	Died of icterus on 5th day.	Positive.
6	"	"	"	"	"	"	"	"
7	July 8 2 cc.	July 14 4 cc.	July 22 4 cc.	Aug. 1	Pure culture 3 cc.	Died of dysentery on 14th day. Autopsy findings negative.	"	

8	July 8 2 cc.	July 14 3 cc.	July 22 4 cc.	Aug. 1	Pure culture 3 cc.	Condition good until 17th day. Killed. Autopsy findings negative.	Died of icterus on 5th day.	Positive.
9	"	"	"	"	"	"	"	
10	"	4 cc.		"	"	"	"	
11	July 10 3 cc.	July 16 4 cc.	July 22 4 cc.	"	"	"	"	
12	"	"	"	"	"	Died of dysentery on 14th day. Autopsy findings negative.	"	
13	"	"	"	"	"	Condition good until 17th day. Killed. Autopsy findings negative.	"	
14	"	"	"	"	"	"	"	Positive.
15	"	"	"	"	"	Died on 3rd day. Autopsy findings negative.	"	

taining 6 to 15 spirochetes in a field were injected into the peritoneal cavity. Table I gives the result of this experiment. 9 of the 15 animals survived for 17 days or longer. 6 died of diarrhea, 1 on the day of injection, 1 the day after, 1 on the 3rd day, 1 on the 8th, and 2 on the 14th day. The autopsy findings were negative. They showed neither icterus nor hemorrhage, and no spirochetes were found in the liver or kidneys under dark-field illumination. The livers of the guinea pigs that died on the 8th and 14th days were emulsified and injected intraperitoneally into the other guinea pigs. Of these animals only the first died of the typical disease, while the others remained healthy. That the spirochetes used for the infection were highly virulent was shown by the control animals, which, having been inoculated at the same time with an equal quantity of pure culture, succumbed after 5 days to the typical disease. Hence it was shown that guinea pigs are highly susceptible to Weil's disease and can be immunized actively by inoculations of a vaccine containing the killed spirochetes or their disintegration products.

In order to ascertain the fate of fresh virulent spirochetes injected intraperitoneally in immunized guinea pigs, we examined every hour the peritoneal fluid of the nine animals mentioned above. 30 minutes after the injection, the spirochetes were few and the majority already degenerated; after 2 hours either no specimen was found or very few; after 10 to 24 hours none were found. We killed the nine guinea pigs and examined them for immune bodies in the blood serum and for spirochetes in the liver and kidneys. The blood serum was positive by Pfeiffer's test, and no spirochetes were found in the liver or kidneys on dark-field illumination. From these experiments it is apparent that the spirochetes gradually disappear in from a few to 24 hours from the peritoneal cavity of the actively immunized guinea pigs; and yet that sometimes virulent spirochetes may still be present after 3 days in the liver and also in the kidneys.

It is, however, remarkable that the blood serum does not contain the immune bodies in demonstrable quantity, even after three inoculations of the vaccine, with Pfeiffer's test, although the guinea pig is protected from infection by virulent spirochetes. However, on injection of the fresh spirochetes the immune bodies appear in the blood in quantities demonstrable by Pfeiffer's test. We shall have something to add on this point later.

Briefly, we could show that spirochetocidal and spirochetolytic bodies develop in the blood of the guinea pig after repeated inoculations which protect the animal from an injection of fresh, virulent spirochetes. We have not yet tested the duration of this active immunity.

Passive immunization is perhaps not possible. We injected as much as 10 cc. of the immune serum into guinea pigs, which was followed after several days by inoculation of active spirochetes. All the animals developed typical infections. No protection was given. But when immune serum and spirochetes were injected at the same time, no infection took place; the guinea pigs were protected from the disease.

Prophylaxis through Protective Inoculation in Man.

We undertook the immunization of a horse through inoculation with vaccine. After demonstrating the appearance of immune bodies in the blood of the horse, we proceeded to the active immunization of man.

At first we tested the vaccine which was employed for immunizing the guinea pigs, but unsuccessfully. Later we prepared a vaccine ten times as strong as the first and with it obtained good results for the first time. Individuals subject to, but who had not had Weil's disease were inoculated subcutaneously, and tests were made for the immune bodies after a definite period.

The body temperature of each person was taken for 1 week. The blood was taken from a vein, and the serum kept in the ice box. The inoculations were made at intervals of 5 days, at the first injection 0.5 cc., at the second 1 cc., and at the third 2 cc. being given. The blood was drawn 10 days after the last injection. Pfeiffer's tests were made with the serum taken before and after the vaccine injection, and with them a control test with isotonic salt solution was made also. The results of the tests are shown in Table II.

From the table it is seen that Pfeiffer's test made with the serum taken before inoculation does not differ from the control test, while that made with the serum drawn after the inoculation shows the presence of immune bodies.

TABLE II.

Experiment No.	Animal No.	Inoculated into peritoneal cavity of guinea pigs.*		No. of spirochetes in peritoneal fluid.		Result.	Autopsy findings.	Spirochetes in liver.
		Serum.	Spirochetes.	After 30 min.	After 2 hrs.			
Control.		Isotonic salt solution 1 cc.	Pure culture (10 in 1 f.) 1 cc.	2–3 in 1 f.	2–3 in 1 f.	Died of icterus on 4th day.	+++	+++
1	1	K. G. serum before inoculation 1 cc.	"	"	1–2 in 1 f.	Died of icterus on 6th day.	+++	+++
	2	K. G. serum after inoculation 1 cc.	"	1–2 in 1 l.	1 in one specimen.	Died on 13th day. No icterus.	+	+
2	3	E.G. serum before inoculation 1 cc.	"	2–3 in 1 f.	1 in 1 f.	Died of icterus on 4th day.	+++	+++
	4	E.G. serum after inoculation 1 cc.	"	2–3 in 1 l.	1 in 1 l.	Died of icterus on 8th day.	++	++
3	5	F.T. serum before inoculation 1 cc.	"	2–3 in 1 f.	1 in 1 f.	Died of icterus on 6th day.	+++	+++
	6	F.T. serum after inoculation 1 cc.	"	1 in 3 f.	1 in 2 l.	Died of icterus on 10th day.	++	++
4	7	N. Z. serum before inoculation 1 cc.	"	2–3 in 1 f.	1 in 2 f.	Died of icterus on 4th day.	+++	+++
	8	N. Z. serum after inoculation 1 cc.	"	None in one specimen.	None in one specimen.	Died of icterus on 12th day.	+	++
Control.		Isotonic salt solution 1 cc.	Liver emulsion (10 in 1 f.) 1 cc.	7–8 in 1 f.	6–7 in 1 f.	Died of icterus on 4th day.	+++	+++
5	9	M. S. serum before inoculation 1 cc.	"	6–8 in 1 f.	5–6 in 1 f.	Died of icterus on 4th day.	+++	+++
	10	M. S. serum after inoculation 1 cc.	"	None in one specimen.	None in one specimen.	Died of icterus on 8th day.	+++	+

Control.		Isotonic salt solution 1 cc.	Pure culture (10 in 1 f.) 1 cc.	2–3 in 1 f.	2–3 in 1 f.	Died of icterus on 3rd day.	++	+++
6	11	I. H. serum before inoculation 1 cc.	"	1 in 1 f.	2–3 in 1 l.	Died of icterus on 6th day.	+++	+++
	12	I. H. serum after inoculation 1 cc.	"	1–2 in 1 l.	1 in a few l.	Died of icterus on 9th day.	++	+
Control.		Isotonic salt solution 1 cc.	Liver emulsion (10 in 1 f.) 1 cc.	1–2 in 1 f.	1–2 in 1 f.	Died of icterus on 6th day.	+++	+++
7	13	M. K. serum before inoculation 1 cc.	"	"	"	Died of icterus on 5th day.	+++	+++
	14	M. K. serum after inoculation 1 cc.	"	2 in a few l.	1 in one specimen.	Died of icterus on 10th day.	++	++
8	15	K. S. serum before inoculation 1 cc.	"	1 in 1 f.	1 in 1 f.	Died of icterus on 6th day.	+++	+++
	16	K. S. serum after inoculation 1 cc.	"	1 in one specimen.	None in one specimen.	Died of icterus on 11th day.	++	+
9	17	A. K. serum before inoculation 1 cc.	"	1–2 in 1 f.	1 in 1 f.	Died of icterus on 5th day.	+++	+++
	18	A. K. serum after inoculation 1 cc.	"	None in one specimen.	None in one specimen.	Died on 15th day. No icterus.	+	+

*The spirochetes were always searched for with dark-field illumination ($\frac{1}{12}$ oil immersion, oc. 3, Leitz).
f. = one field.
l. = from one side to the other of the cover glass (65 to 70 fields).

In the first instance one sees that as regards Pfeiffer's test the spirochetes in the peritoneal fluid are identical with the serum taken before the inoculation and the salt solution; while the blood serum taken after the inoculation yields after 30 minutes scarcely any spirochetes, and after 2 hours merely a degenerate specimen in a preparation. The guinea pig died, however, after 13 days, without icterus; that is, 7 to 9 days later than the control animal and the animal with serum taken before the inoculation. The internal organs showed some hemorrhage and a few spirochetes in the liver. Hence the existence of immune bodies in the blood is shown, although still insufficient to prevent all infection. The ninth animal gave a similar result. The other animals did not show as high a grade of immunity as these two. The difference in survival between the immunized and the control animals amounts in several cases, respectively, to 7, 8, and 10 days, in 2 cases to 5, in 3 cases to 4, and in 1 case to 3 days. The difference of 4 days indicates the lowest limit of the incomplete immunity. If the serum contains no immune bodies, as is the case in the blood serum of normal individuals, the experimental animal succumbs to the typical disease on the same day as the control, or at the most, a day or two earlier or later. A difference of 3 days is seldom seen. If, therefore, the experimental animal dies of the typical disease 4 days later than the control animal, we may conclude that the blood serum of this animal contains a certain quantity of immune bodies. The greater the quantity of immune bodies in the blood serum, the longer is the time of incubation for the guinea pig, and finally when complete immunity is attained, the animal no longer becomes ill after injections. This phenomenon of gradually increasing immunity can readily be followed by Pfeiffer's test of the blood drawn each day after the 6th or 7th day of the disease. This experiment thus indicates that the immune bodies appear in the blood after inoculation.

The question arises as to whether or not this incomplete immunity is able to protect the organism against infection with *Spirochæta icterohæmorrhagiæ*. As we are not in a position to decide this question by the injection of the pathogenic cause directly into man, we are obliged to draw our conclusions indirectly from the immunity reaction of patients and from animal experimentation.

This state of incomplete immunity is observed in patients after the 8th to the 11th day of the disease. In this stage (the second stage according to Inada and Ido, or the icteric period according to Oguro) the experiments of infection with the blood of the patient are, as a rule, negative. The spirochetes in the blood have all been killed by the immune bodies. After the 8th or 9th day of the disease, there are likewise no, or very few degenerated spirochetes in the liver. The spirochetes in the liver have already been destroyed by the immune bodies in the incomplete state of immunity. It is not possible to demonstrate immune bodies by Pfeiffer's test within a week after the onset of the disease. The experimental animals die as rapidly as the controls. Nevertheless, one recognizes from the infection experiments conducted with the blood of patients, that a certain quantity of the immune bodies must have appeared in the blood. The intraperitoneal injection of 2 or 3 cc. of blood into the guinea pig produces the disease in typical form up to the 4th day in 100 per cent of cases (26 cases, all positive), on the 5th day in 91.6 per cent (only 1 case among 12 being negative), on the 6th day in 85.7 per cent (only 2 negative cases among 14), on the 7th day in 50 per cent (4 negative and 4 positive cases). These gradually decreasing percentages of positive results can be explained only by the appearance of the immune bodies. The almost completely negative findings in the liver on the 7th day of the disease indicate the appearance of immune bodies even inside of 1 week. Nevertheless, up to the 7th day we cannot prove the existence of immune bodies in the blood serum by Pfeiffer's test. But, we may conclude that the degree of partial immunity described above suffices to protect the organism against infection from the spirochete of Weil's disease.

By means of these animal experiments it was possible to establish the fact that three inoculations protect the guinea pig against infection with the spirochete; but notwithstanding this active immunity, it is not possible to demonstrate by Pfeiffer's test immune bodies in the blood serum of the guinea pig (Table III).

We inoculated six guinea pigs, giving each three injections of 1 cc. of vaccine after an interval of 5 days, and then injected intraperitoneally into three guinea pigs pure cultures of *Spirochæta ictero-*

TABLE III.

Animal No.	Date and quantity inoculated.			Spirochetes injected.	Result.	Autopsy findings.	Spirochetes in liver.	Result.
	I	II	III					
1	Jan. 20 1 cc.	Jan. 25 1 cc.	Jan. 29 1 cc.	Pure culture (30 in 1 f.) 1 cc.	Died of dysentery on 19th day.	–	–	–
2	"	"	"	"	Died of dysentery on 16th day.	–	–	–
3	"	"	"	"	Died of dysentery on 18th day.	–	–	–
Control.				"	Died of icterus on 4th day.	+	+	+
1	Jan. 22 1 cc.	Jan. 27 1 cc.	Jan. 31 1 cc.	Not injected.	Killed Feb. 9 to get blood serum.			
2	"	"	"	"	"			
3	"	"	"	"	"			

Pfeiffer's test.

Animal No.	Inoculated into peritoneal cavity of guinea pigs.		No. of spirochetes in peritoneal fluid.		Result.	Autopsy findings.	Spirochetes in liver.	Result.
	Serum.	Spirochetes.	After 30 min.	After 2 hrs.				
1	V_1 serum 1 cc.	Pure culture (10 in 1 f.) 1 cc.	2–3 in 1 f.	2–3 in 1 f.	Died of icterus on 5th day.	+++	+++	–
2	V_2 serum 1 cc.	"	"	1 in 1 f.	"	+++	+++	–
3	V_3 serum 1 cc.	"	"	2 in 1 f.	Died of icterus on 4th day.	+++	+++	–
4	Isotonic salt solution 1 cc.	"	"	2–3 in 1 f.	"	+++	+++	–

hæmorrhagiæ. Three other animals were killed and their serum was tested by Pfeiffer's method. The three guinea pigs into which the pure cultures were injected died of diarrhea after 16 to 19 days, without showing the typical disease. The autopsy findings were completely negative in all. No spirochetes could be found in the organs. 4 days later, the control animal died of the typical disease. In the serum of the three guinea pigs that were killed no trace of immune bodies could be demonstrated by Pfeiffer's test. The experimental animals died of icterus and hemorrhage as soon as the control animal. From these experiments it is evident that subsequent to the inoculation, the serum contains immune bodies, but while the small number makes it impossible to prove the existence of the organism by Pfeiffer's test, it is sufficient to prohibit a proliferation of the spirochetes in the body of the guinea pig. After the injection of virulent spirochetes into the inoculated guinea pig, the immune bodies appear in the blood. From the clinical findings and the animal experiments we may conclude that a quantity of the immune bodies in the blood, which cannot be proved by Pfeiffer's test, acts prophylactically against infection with *Spirochæta icterohæmorrhagiæ.*

The secondary effects of the inoculation are scarcely noticeable in man. In the guinea pig we often observed fever following an injection, but this never occurred in man. In two cases there was slight headache and general lassitude, but these manifestations were negligible and disappeared completely in from 1 to 3 days. The chief complaint was of pains at the site of injection. One often sees local swellings and slight redness, but these disappear after 24 hours. The pains also are not severe.

It is not known how long the partial immunity continues, but it is probable that it lasts from 6 months to a year.

DISCUSSION AND SUMMARY.

We have already described briefly the portals of entry and of excretion of the pathogenic spirochetes.[2] We may mention here

[2] Inada, R., Ido, Y. Hoki, R., Kaneko, R., Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

that we have twice prevented epidemics by disinfection of the ground and the removal of the inundated water in certain places in coal mines. In one mine 19 out of 50 workmen, and in another 9 out of 30 workmen came down with Weil's disease in about 2 weeks.

We have already pointed out that the period during which the pathogenic spirochetes are excreted in the urine continues, as a rule, for 40 days, and that we must, therefore, apply disinfection for at least 40 days after the first appearance of the disease. Lately we have found that in 21 cases out of 24 the spirochetes were excreted in the urine for 40 days, in one case until the 42nd day, in one case until the 45th day, and in still another case until the 63rd day.

Another important fact concerning the prophylaxis which has been brought out is that both house and ditch rats (brown) carry virulent *Spirochæta icterohæmorrhagiæ*, the causal spirochete of Weil's disease, in their kidneys. Miyajima[3] has reported that field rats have the pathogenic organisms in their kidneys; he will report these findings in detail later. The spirochetes which he described are less virulent than ours. On his advice we have carefully examined house and ditch rats in the city and rats in the coal mines of Kyushu, where Weil's disease prevails, and found that 39.5 per cent carried highly virulent pathogenic spirochetes in their kidneys, thus confirming Miyajima's experiments. The kidneys were examined microscopically under the dark-field microscope, and in the cases in which we did not find the pathogenic spirochete, we made inoculations into guinea pigs. Thus we found *Spirochæta icterohæmorrhagiæ* microscopically in the kidneys or in the urine in 32.4 per cent, and by means of inoculation in 7 per cent, making a total of 39.5 per cent carrying the pathogenic organisms, out of a total number of 86 rats examined. In some instances, rats were made to bite guinea pigs and in two instances caused Weil's disease. Among fifty-five patients in our clinic, twelve were cooks; and in Europe many cases arise among butchers—indicating the relation of the disease to rats. Moreover, during the present year we observed two patients who acquired Weil's disease, one in 1 week, the other 8 to 9 days after they had been bitten by rats. These facts point to a relation be-

[3] Reported at the April, 1916, meeting of the Fellows of the Kitasato Institute for Infectious Diseases.

tween Weil's disease and rats. The infection is transmitted probably from rats to man by means of the urine of the rats, directly or indirectly. On the injection of 0.1 gm. of rat urine which contains *Spirochæta icterohæmorrhagiæ* into the peritoneal cavity of guinea pigs, the infection arises, while the injection of the liver or the blood of the rats into guinea pigs does not produce the typical disease.

The finding that the kidneys of rats contain the pathogenic organisms of the disease is important from the point of view of prophylaxis. The large number of rats in the trenches of the European battle-fields suggests the possibility that many cases of Weil's disease may arise. We shall report on this point in more detail later.

THE SERUM TREATMENT OF WEIL'S DISEASE (SPIROCHÆTOSIS ICTEROHÆMORRHAGICA).

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Although Weil's disease has been recognized since antiquity and its mortality has been high, treatment up to the present time has been purely symptomatic, as the causative agent was not known. But with the discovery of the cause, we have now entered upon the study of the specific therapy. In the course of their discovery of the causative agent, Inada and Ido[1] found in the blood serum obtained from recovered cases of the disease an immune body which acted as a solvent and destroyed the spirochetes contained in the blood and the liver of the guinea pigs under experimentation; on the basis of this work they advanced the hypothesis that serum therapy should be effective in Weil's disease.

In his experimental investigation Ido obtained remarkable results with convalescent serum and active serum from immunized goats. He demonstrated that the immune serum, if injected prior to the appearance of icterus, is capable of inhibiting the disease in all cases. This work forms the basis of the serum therapy of Weil's disease in man. On the other hand, experiments undertaken with salvarsan, as already indicated elsewhere,[2] were not followed by comparable results. For these reasons we have been led to employ antiserum in the treatment of Weil's disease since August, 1915; and we were in a position to report the results of our work as early as October, 1915.

[1] Inada and Ido, A Report on the Discovery of the Causal Organism (a New Species of Spirochæta) of Weil's Disease, *Tokyo Ijishinshi*, 1915, No. 1908.

[2] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., The Etiology, Mode of Infection, and Specific Therapy of Weil's Disease (Spirochætosis Icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

Up to the present time serum therapy has not been widely employed in the spirochetal diseases. Since the serum therapy of readily relapsing spirilloses leads to the formation of a serum-fast strain, it has been claimed that salvarsan would be more suitable for treatment in these cases. But in Weil's disease a serum-fast strain does not occur. Spirochetolytic and spirochetocidal immune bodies, however, develop in the blood, and the immunity lasts for several years, the antibodies having in one case been unmistakably detected 8 years after recovery. Relapse apparently does not occur in Weil's disease. We have never found a case of it, although Fiedler recognizes it. Inada and Ido have given the term "after fever" to the fever which occurs on the 14th or 15th day from the onset of the disease, sometimes on the 13th to the 16th day, less frequently on the 12th to the 17th, and which continues for several days, often from 7 to 10. But this cannot be considered a relapse in the real sense of the word, because at this stage of the disease we do not find the spirochetes in the blood, or in any organs but the kidneys.

We employed first serum from recovered cases and later serum obtained from actively immunized horses. As human convalescent serum is not procurable in large quantities, a horse was immunized with *Spirochæta icterohæmorrhagiæ.* The goat also can be readily immunized, but is too small an animal to yield large amounts of serum. At the present time we possess two actively immune horses, which are able to take an injection of 800 cc. of pure culture, containing 30 spirochetes per field, under dark-field illumination ($\frac{1}{12}$ oil immersion, No. 3 ocular, Leitz).

The serum which we now have is far more potent than that obtained at the beginning. 0.01 cc. of the present serum injected simultaneously with a pure culture into the peritoneal cavity protects a guinea pig weighing 200 gm. against infection from 1 cc. of pure culture (10 spirochetes per field). In the treatment of the patients considered here, the serum was of weaker concentration, as much as 0.5 cc. of the serum being required to protect a guinea pig against infection from a similar dose of culture. Inasmuch as our most potent serum was obtained only toward the end of 1915, the opportunity to test its action did not arise, as we have had no cases of Weil's disease since that time.

To the serum obtained from horses, 0.5 per cent carbolic acid was added and it was kept in the ice box for 1 month. It was then tested for tetanus toxin and carbolic acid content, and also by cultures, to establish sterility. We employed serum taken on July 12, August 28, September 19, and October 11 from Horse 1. 35 patients in all received the treatment; 23 in the clinic and 12 cases occurring in a coal mine. Of these patients, 6 were treated with human convalescent serum, 2 with both horse and human serum, and the remaining 27 patients with horse serum alone. As the titer of the horse serum about equalled that of the human serum, the cases treated will be discussed jointly.

Dose of Serum Injected.—The initial daily dose for 3 days was 10 cc., but our experience derived from a number of cases shows that this dosage is ineffectual. For that reason we employed gradually larger doses, until finally we injected 60 cc. in 24 hours. This quantity of serum was given in three amounts, with intervals of from 5 to 6 hours. Of late, we have made intravenous injections with good results. It was feared in the beginning that the toxin liberated by lysis of the spirochetes would prove harmful to the organism, but this fear proved to be unfounded.

The day on which serum treatment was instituted is shown in Table I.

TABLE I.

Day of illness.	No. of cases.
2	4
3	4
4	5
5	6
6	8
7	3
8	5

In 24 of the cases icterus was present at the beginning of the serum treatment; 11 cases showed no icterus when the treatment was begun; and in 5 there was no icterus during the course of the disease. As the serum, according to our experience, is mainly spirochetolytic and spirochetocidal in action, and its antitoxic effect has not yet been

demonstrated, it is recommended that it should be employed as early as possible after the onset of the disease,—if possible, before the 5th day of illness, when there are probably many spirochetes present in the organs. The distribution from the point of intensity of the disease was as follows: 17 severe cases, 17 moderately severe, and 1 mild case.

The criteria employed to determine the result of the serum therapy were:

1. The rate of mortality.
2. The influence of the serum upon the spirochetes in the circulating blood.
3. The effect of the serum on an earlier development of antibodies.
4. The number of spirochetes found in the organs after death, when serum treatment had been employed.
5. The influence upon the symptoms and the progress of the disease.

Of these criteria, special emphasis has been placed upon Nos. 2 and 3, inasmuch as they afford definite objective phenomena for judging the effect of the serum treatment, and are valid even if the number of patients is small, while for the other criteria a large number of observations is required. The difficulty of determining the effect upon the symptoms and the course of the disease in individual cases increases with an early use of the serum, as in that case it is not easy to decide whether the slight illness is attributable to the serum, or whether the disease as such was of a mild character. The prognosis in most cases is determined at the height of illness, or on the 7th or 8th day from the onset.

Mortality in Weil's Disease.

Of the 35 patients who received the serum treatment, 7 died. Of this number, 2 showed complications, 1 developed suppurative meningitis on the 27th day of the disease, and another patient who had a suppurative process died after 2 months. This leaves only 5 cases in which the cause of death is attributable to Weil's disease itself rather than to a complication. Of these, one patient, who was admitted in a moribund condition and died on the following day, has not been included. Omitting these cases, the mortality was found to be 11.4 per cent. If we exclude also the 12 patients in the coal mine and include only the 23 cases treated at the clinic, the mortality is 17.3 per cent.

According to Oguro, the mortality of Weil's disease in Japan is 40 per cent, according to Nishi, 48 per cent; and according to Inada, 30.6 per cent. As we are not in possession of definite data regarding the death rate among the patients in the coal mine, we have excluded these cases and have compared only the mortality of patients in our clinic, treated with serum or otherwise, with the general statistics. The mortality in our clinic up to last year was 30.6 per cent. With the beginning of the serum therapy, it will be seen that the mortality is reduced almost to two-thirds. It must, however, be stated that we have, during the past year and since the discovery of the causative agent, accepted a relatively large number of patients, and among these there were probably a number who had the disease in a mild form.

In view of the reduction in the mortality rate, at the moment, we shall limit our claim to the statement that the administration of the serum has a marked effect in Weil's disease, and we shall await the results obtained from a large number of cases before making a more positive assertion.

The Behavior of Spirochæta icterohæmorrhagiæ in the Circulating Blood after Serum Injection.

We desire to present in brief form the result of infection experiments carried out with the blood of patients who received no serum treatment. Table II shows the result of this investigation which comprises 68 cases, arranged according to the day of illness.

TABLE II.

Infection Experiments with Blood from Weil's Disease.

Day of illness.	No. of cases.	Results.		Per cent of positive results.
		Negative.	Positive.	
Up to 4	26		26	100.0
5	12	1	11	91.6
6	14	2	12	85.7
7	8	4	4	50.0
8	4	4	0	0
9	1		1	100.0
12	1	1		0
18	1	1		0
19	1	1		0

It will be seen that in all cases up to the 4th day of illness, the result was positive; of the experiments undertaken on the 5th day 91.6 per cent were positive; on the 6th day, 85.7 per cent; on the 7th, 50 per cent. From the 8th day on, of blood taken from 8 patients, only one, that taken on the 9th day, proved to be positive.

In order to determine whether the dose of serum employed is capable of killing and dissolving the spirochetes in the circulating blood of the patient, blood was taken, before the injection of serum, from the arm vein of the patient and injected intraperitoneally into guinea pigs; and, similarly, another sample of blood was drawn after the injection of the serum. If, then, the guinea pig receiving the blood drawn before the injection of serum became typically ill, while that receiving the blood drawn after serum had been injected into the patient remained well, the last drawing of blood having been made on the 4th day of the disease, we may conclude that, in the latter case, the spirochetes in the circulating blood had been destroyed by the serum. When the blood is taken on the 5th or 6th day, the determination is somewhat more difficult, inasmuch as blood injected on the 5th day gave negative results in 8.4 per cent, and that drawn on the 6th day was negative in 14.3 per cent of the cases. It is possible to estimate the spirochetocidal action of the serum from the difference in the percentages of infectability of the injected blood (Table III).

TABLE III.

Infection Experiments with Blood Drawn from Patients with Weil's Disease before and after the Injection of Serum into the Patient.

No.	Day of illness on which serum treatment was given.	Results.		Quantity of serum injected.	Time between serum injection and drawing of blood.	Day of disease after serum treatment.	Positive results.	
		Before serum injection.	After serum injection.				With serum treatment.	Without serum treatment.
				cc.	*hrs.*		*per cent*	*per cent*
1	2	+	+	10	30	3		
2	2	+	−	40	6	3	25.0	100.0
3	2	+	−	60	24	4		
4	2	+	−	40	24	4		
5	2	+	−	60	24	5		
6	3	+	+	20	24	5		
7	3	+	±	60	24	5		
8	3	+	−	60	24	5		
9	4	+	+	20	5	5	50.0	100.0
10	4	+	+	10	7	5		
11	4	+	−	80	12	5		
12	5	+	−	20	5	5		
13	3	+	±	40	4th day.	6		
14	4	+	−	35	22	6		
15	5	+	−	40	13	6		
16	5	+	+	10	7	6		
17	5	+	+	10	24	6	71.4	77.7
18	5	−	−	60	17	6		
19	5	+	+	60	12	6		
20	6	+	+	10	3	6		
21	6	−	−	10	7	6		
22	6	+	−	10	12	7		
23	6	+	±	40	15	7	33.3	100.0
24	6	+	−	80	7	7		
25	7			60				
26	8			60			0	0
27	8	−	−	10	7	8		

Table III shows that in four cases in which human blood not subjected to serum treatment was capable of infecting guinea pigs, when drawn on the 4th day of the disease after serum treatment had been given, the infection was positive in only a single case. The

ratio of infection of the guinea pigs with blood drawn after the administration of serum is, therefore, 25 per cent. If we compare this figure with the percentages of infection (100 per cent) found in guinea pigs receiving blood taken before serum treatment, a vast difference is apparent. This difference in results justifies us fully in concluding that the blood taken from patients after they have received serum treatment is no longer capable of infecting guinea pigs. This means either that the serum has destroyed the spirochetes in the circulating blood, or that the spirochetes have lost their virulence and are no longer capable of producing an infection. The positive result in a single case is to be attributed to the insufficient dosage of the serum, only 10 cc. having been injected in the case in question. On the 5th day the percentage of guinea pigs infected after serum treatment was 50 per cent; without serum treatment, 100 per cent. The positive result in 3 cases is also due to the insufficient quantity of serum administered, as only from 10 to 20 cc. had been injected. In one case the infection experiment proved negative even when 20 cc. of the serum had been administered.

On the 6th day the percentage of illness of the guinea pigs, subsequent to serum treatment, was 71.4; without serum treatment, 77.7. Among the patients there was one who received 60 cc. of the serum, and whose blood was still capable of infecting guinea pigs after a period of 20 hours. It is difficult to say why the spirochetes in the blood were not destroyed by the serum.

On the 7th day of illness, 33.3 per cent of guinea pigs infected with blood taken after serum treatment became ill, and 100 per cent without serum treatment. Here we have the blood of a patient which caused an infection in one guinea pig, while another animal remained well. This case has been included among the positive results.

From these results we may conclude that the horse serum which we have prepared, administered in suitable doses, is capable of destroying completely the spirochetes contained in the circulating blood, by the injection of 40 to 60 cc. of serum in from 5 to 6 and up to 24 hours. A quantity of 10 to 20 cc. of the serum did not suffice to kill the spirochetes.

The Influence of the Serum Treatment upon the Appearance of Antibodies in the Blood.

As we have stated elsewhere,[3] the antibodies in Weil's disease are not demonstrable by Pfeiffer's method in the blood serum of patients inside of 1 week from the onset of illness. Beginning with the 8th or the 11th day, antibodies make their appearance in the blood, although they are at first weak; by the 12th or the 13th day they are fully active. They are rarely active as early as the 8th day. The time of development of the antibodies in the blood seems to be an individual characteristic, and differs according to the severity of the disease. We have designated as incomplete, with respect to antibody formation, those cases in which the infected animals (tested by Pfeiffer's method) outlived the control animals by 4 days or longer. A difference of 3 days in the life of the experimental and the control animals should be regarded as an indication of incomplete immunity, but we have taken 4 days as a conservative limit.

TABLE IV.

The Period of Development of Antibodies in the Blood of Patients with Weil's Disease.

Day of illness.	No serum treatment.				Serum treatment.			
	Complete development.	Incomplete development.	Negative.	Total.	Complete development.	Incomplete development.	Negative.	Total.
6			3	3	1	1	7	9
7			5	5		2	3	5
8	2	2	6	10	3	8	7	18
9		3	4	7	3	1	2	6
10		1	2	3	5	5	2	12
11	1		2	3		1	1	2
12	3		1	4	3			3
13	43			43	7			7

One patient treated with the serum showed complete development of antibodies in the blood on the 6th day of illness. This phenomenon never occurs with the blood of patients who have not received the serum. As shown in Table IV, in a large percentage of the patients

[3] Ido, Y., Hoki, R., Ito, H, Wani, H., The Prophylaxis of Weil's Disease (Spirochætosis Icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

receiving serum treatment complete development of antibodies could be demonstrated as early as the 9th or 10th day, while this condition appeared only on the 11th or 12th day when no serum had been administered. If we also include the cases of incomplete development of antibodies, we are justified in concluding that when serum treatment is given, the antibodies make their appearance in the blood earlier than in those cases where no serum is administered. As far as the dosage of serum in relation to the development of antibodies is concerned, this is difficult to estimate, for the intensity of the disease and the individual circumstances must always be taken into account, but speaking generally it may be said that development proceeds more rapidly with a large dose of serum.

The Influence of Serum Treatment upon the Spirochetes in the Body.

As Kaneko and Okuda[4] have discussed this point in detail, we shall present only a brief outline here. It may be said, in general, that the number of spirochetes found at autopsy in the bodies of patients who had received serum treatment is much less than that found in patients ill for an equal period of time who received no serum; and, moreover, the spirochetes in the first instance show a high degree of degeneration. This statement is particularly true of the kidneys and the cardiac muscles.

The Influence of Serum Treatment upon the Symptoms and the Course of the Disease.

Whether or not the serum has an appreciable effect upon the fever, icterus, and hemorrhagic diatheses can be determined only by more extensive experience. Up to the present time, we have observed no significant effect, except that with serum treatment the hemorrhages are less frequent than without serum treatment. The "after fever," on the other hand, seems to occur more frequently when serum has been given. Serum therapy has no definite effect upon the excretion of spirochetes in the urine, although the period of excretion of the

[4] Kaneko and Okuda, The Pathological and Anatomical State of the Lymph Glands in the Early Stage, and the Distribution of the Spirochæta in Them, *Tokyo Ijishinshi*, 1915, No. 1940–1945.

living spirochetes seems to be shorter where large doses of serum have been employed.

Secondary Manifestations.

We have observed few secondary effects. Following the injection of serum, no fever occurred. In one case only was there a complaint of headache, in another of tinnitus, and in still another there appeared an exanthem at the site of injection. These symptoms which were all mild in character disappeared completely after 1 or 2 days. Frequently complaint was made of slight pains at the site of injection, but these also disappeared on the following day.

Spirochetocidal and Spirochetolytic Action of Serum.

We have recently observed a case in which we were able to demonstrate experimentally upon guinea pigs in a conclusive manner the spirochetocidal and spirochetolytic action of serum from Weil's disease. The patient was a man of 65, who was taken ill suddenly on May 1. He was admitted to the clinic on the 4th day of the disease. A large number of *Spirochæta icterohæmorrhagiæ* were found in the blood under dark-field illumination; *i.e.*, 14 to 16 on a cover slip (60 to 70 optical fields). In no other case have we found the spirochetes so numerous, for as a rule the organisms are not readily found in fresh blood preparations. The number of spirochetes found before and after serum injection was as follows:

May 4, 2.00 p.m. Admitted to clinic, 4th day of illness. Spirochetes in the blood, 14 to 16 per field, as described above.

3.00 p.m. 17 cc. of serum, very active (from Feb. 26), subcutaneously.

6.00 p.m. Spirochetes 14 to 16.

7.00 p.m. Spirochetes 10 to 16.

8.00 p.m. 20 cc. of serum, intravenously.

10.00 p.m. No spirochetes in 2 preparations.

12.00 m. No spirochetes in 3 preparations.

1.30 a.m. No spirochetes in 1 preparation.

At 1.00 a.m. we injected 2 cc. of the blood of the patient into the peritoneal cavity of a guinea pig. Up to the time of writing, May 14, the animal has remained well. At 8.30 a.m., 3 cc. of the blood were injected into the peritoneal cavity of another guinea pig. This animal also has not become ill.

2 hours after intravenous injection, we were unable to find any spirochetes. This result in the experimental animal is in entire accord with the spirochetocidal and spirochetolytic action of the serum observed in human beings.

SUMMARY.

Horses immunized with cultures of *Spirochæta icterohæmorrhagiæ* yield an immune serum having therapeutic properties.

With rare exceptions the serum destroys completely the spirochetes contained in the circulating blood.

The development of antibodies is promoted by the serum injections.

The number of spirochetes in the organs is reduced by the treatment.

Secondary manifestations due to the serum are slight and disappear promptly.

The ultimate effects of the serum treatment on the symptoms and final outcome of the disease have still to be determined.

EXPERIMENTAL OBSERVATIONS ON THE PATHOGENESIS OF GALL-BLADDER INFECTIONS IN TYPHOID, CHOLERA, AND DYSENTERY.

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The fact that the chronic carrier in the typhoid group of diseases is a result of infection is becoming of more and more importance in epidemiology. In most cases the microorganisms are known to be carried in the gall-bladder or gall-passages. In cholera, the infection seems to produce nearly the same result. At least semichronic intestinal carriers have been found and their occurrence has been shown by Kulescha (1), Greig (2), Schöbl (3), and others to be due to infection of the gall-bladder. This discovery is the most important recent addition to the pathology and epidemiology of the disease. In the case of bacillary dysentery, fewer cases have been studied from this point of view, but intestinal carriers have been reported, and in a few instances dysentery bacilli have been recovered from the bile (4), and analogy and experiment suggest a similar set of circumstances. In short, in this whole group of diseases, one of the most important problems of preventive medicine seems to be the prevention and cure of gall-bladder and gall-passage infections.

The subject has been approached from the experimental side by making use of gall-bladder infections in the rabbit. In some respects, at least, the lesion in the rabbit is a counterpart of that in man, and it seems probable that, in working out means of preventing and curing carriers, the experimental lesion in the rabbit will be an important factor. The present paper deals with experimental observations on the mechanism of gall-bladder infection in typhoid, concerning which our knowledge at present is still uncertain; the mechan-

ism of infection in cholera and dysentery by a portal system septicemia is suggested, and the antiseptic properties of rabbit bile are emphasized.

Mechanism of Gall-Bladder Infection in Typhoid.

Logically, as has often been stated, the gall-bladder lesion might be due to descending infection of the bile from the liver, to an ascending infection of the bile from the intestines, or to a transverse infection through the gall-bladder wall from the blood-vessels. The theory of descending infection from the liver was most generally accepted up to 1908 when Koch (5) and Chiarolanza (6) claimed to have proved that transverse infection through the gall-bladder wall is the usual method. Their views are more or less accepted at present (7). The theory of ascending infection from the intestines was early abandoned on account of the rarity of clinical gall-bladder infections with *Bacillus coli* and on account of positive results following intravenous injections in animals with or without ligation of the common duct.

It is desirable to settle this question if possible, because from the point of view of therapeutic attack, at least for prophylaxis, it makes a great deal of difference whether the infection occurs from the bile or from the blood vessels of the bladder wall. The writer is convinced that Koch and Chiarolanza's work is not conclusive and believes that we should go back to the theory of descending infection from the liver as an explanation of the usual method of infection. Occasionally, of course, infection might occur in any one of the three ways, but it seems reasonable that there is some regular method.

HISTORICAL.

The observations on which the theory of descending infections rests date back to our early knowledge of the elimination of microorganisms by the liver. Apparently the first work done was that of Fütterer (8), who, in 1888, in connection with the study of cases of typhoid with cholecystitis, reported that he injected a culture of *pyocyaneus* into the left ventricle of rabbits and recovered the organism in the gall-bladder at least 1½ hours later. In 1895 Fütterer (9) reported that he made a gall-bladder fistula in a dog and recovered *Staphylococcus aureus* from the bile 40 minutes after subcutaneous injection. He em-

phasized the importance of the liver in freeing the blood from microorganisms, and also referred to bacilli in the gall-bladder as a possible cause of relapse in typhoid, but of course, at that time, the possibilities of chronic cholecystitis in the spread of the disease were not realized. In 1899 Fütterer (10) recorded further experiments with dogs in which he established a common duct fistula and gave a portal vein injection of a suspension of *Bacillus prodigiosus* and recovered the organism from the bile 2 minutes after injection.

The work of Fütterer was confirmed by several workers, especially by Biedl and Kraus (11) who, in 1896, took up the question of elimination of bacteria by the bile in the course of a general study of the elimination of microorganisms by the liver, kidneys, and salivary glands. They made common bile-duct fistulas in dogs and recovered staphylococci after intravenous injection in 13 to 45 minutes. In 1905 Doerr (12) made a more particular study of the typhoid gall-bladder problem and in investigating the method of infection tied the cystic duct in rabbits; after several days he gave an intravenous injection but obtained no infection. However, when he ligated the common duct and gave an intravenous injection, an infection of the gall-bladder resulted.

These observations established the excretory powers of the liver and seemed to afford a natural explanation for gall-bladder infections in a septicemia like typhoid. In 1908, however, Koch and Chiarolanza published their articles in which they claimed to have demonstrated that infections occur through the gall-bladder wall and not through the bile. Koch drew his conclusions from the histological picture in a human case of typhoid cholecystitis in which he found emboli of bacilli in the folds of the mucous membrane. Chiarolanza tied the cystic duct and immediately injected typhoid bacilli intravenously and recovered them from the gall-bladder. He concluded that the usual method of infection was through the gall-bladder wall. However, as will be shown later, his experiments form a slender basis for adopting this theory, although many recent articles assume that the gall-bladder wall infection method is the proved method of infection (7).

Technique.

A number of experiments have been made by the fistula method. At first large Belgian hares, weighing 10 kilos, were used in order to make the operation easier, but it was found that smaller animals, even guinea pigs, can be easily used. Under ether anesthesia, a median incision is made from the ensiform cartilage to below the umbilicus, the stomach and duodenum are drawn down by an assistant, putting the common duct on the stretch; the mesentery over the duct is cut, avoiding the vessels, and a threaded aneurysm needle is passed under the duct about 1 inch from the duodenum. The thread serves

to cut off the bile while the capillary tube is being inserted and to tie the tube in place. The thread is put on the stretch by an assistant to constrict the duct, a small transverse incision is made below the constriction with a small pair of scissors, the capillary tube, which rapidly fills with bile, is inserted, the thread is tied on the neck of the tube, the rubber tubing attachment is brought out by a small opening in the right side of the abdominal wall, and the wound is closed. The rubber tube is attached to a glass tube in a two-holed stopper and the stopper is inserted in a graduated centrifuge tube. Bile soon begins to drop and accumulates at a rate of 1 cc. every 5 to 8 minutes, depending on the size of the animal. The capillary and rubber tube hold about 0.5 cc. of bile. If care is used in inserting the tube and in tying the duct securely, no blood enters the duct and brilliant green bile can be obtained in large quantities. With proper asepsis few contaminations are found in the plated bile. After injection, the bile is plated in 0.5 and 1.0 cc. quantities, and the colonies are counted after 48 hours' incubation. Most of the typhoid, cholera, and dysentery colonies were typical, but in case of doubt, they were identified by staining, by the double sugar tube, and by agglutination. Mesenteric injections were made into the veins of the appendix; some bleeding occurs when the needle is withdrawn, but it stops spontaneously and no gangrene of the intestine follows.

Most of the animals were about the same weight (3 kilos). The time of collection of bile was limited to about 1 hour, as it was found that cultures taken after 1 hour up to 24 hours were almost always sterile, even when many bacilli came through in the 1st hour.

The writer realizes, of course, that bacilli may appear in the bile from foci in the liver after the 1st hour, but the short observation time brings out points of importance and there is a limit to the experimental method on account of emaciation of the animal and infection of the field of operation. Moreover, the process of elimination seems to resemble that of filtration, and Bull (13) has shown that the blood after intravenous injection is usually sterile in less than an hour. Most of the animals were chloroformed in 1 or 2 days but two animals lived over a week before they were killed. Altogether twenty-four rabbits and two guinea pigs were successfully operated on.

EXPERIMENTAL.

If the theory of descending infection is accepted as a working hypothesis and we attempt to test it experimentally it is evident that two conditions must be met; first, the bacilli must get into the bile, and second, they must be able to multiply in the bile. Infection of the rabbit's gall-bladder with typhoid bacilli has been produced many times by intravenous injection since the first work on this subject by Blachstein (14) and Welch (15) in 1891, but the results are uncertain and on an average not more than 50 per cent of inoculations are successful. In order to secure 100 per cent of infections which are necessary in therapeutic tests many authors have used the direct inoculation of the gall-bladder. This method is almost always successful, but it introduces a new element of traumatism and escape of blood into the gall-bladder and throws no light on the ordinary method of infection. If an explanation of the failure of intravenous injections to produce 100 per cent of infections can be found it may furnish a clue to the solution of the whole problem.

Presence of Bacilli in the Bile.—In the first set of experiments, the cystic duct was tied in order to prevent the possible entrance of organisms from the gall-bladder; a common duct fistula was then made, which, incidentally, prevented any organisms coming from below, and an injection was made into a mesenteric vein in order to favor concentration of the organisms in the liver. 1 cc. of a 24 hour broth culture was used for injection; the bile was collected every 5 to 15 minutes and poured into mannite media or over the surface of a double sugar tube, or Loeffler's media, depending on the organism used. In this way I obtained positive evidence of the presence in first specimens of bile of typhoid, paratyphoid A, and dysentery Y bacilli, and cholera vibrios. These experiments convinced me that microorganisms regularly enter the bile from the liver if they are present in the blood in sufficient numbers. In the rest of the experiments the cystic duct was not tied and the bile was plated in order to obtain quantitative results.

Relation of the Number of Bacilli Injected to the Number of Bacilli Appearing in the Bile.—It is known that, in general, larger intravenous doses produce more positive results in the gall-bladder and

several experiments have been carried out to determine what dose is necessary for bacilli to appear in the bile. Fistulas were established and different doses of a 24 hour broth culture of a recently isolated typhoid bacillus were injected into the ear vein. Bile was collected and plated, and the colonies were counted after 48 hours' incubation (Table I).

TABLE I.

Number of Colonies in Plated Bile after Injection into Ear Vein of Different Quantities of Typhoid Bacilli.

Bile.	1 cc.	2 cc.	3 cc.
cc.			
0.5	—	—	—
0.5	—	—	50
0.5	—	—	25
0.5	—	—	6
0.5	—	—	10
1.0	—	1	4
1.0	—	—	2
1.0	—	—	—
1.0	—	—	3
1.0	—	—	2
Total colonies in 7.5 cc. of bile.....	0	1	102
Time of collection in min..........	65	45	45

These experiments show that a fairly large dose is necessary for bacilli to appear in the bile in any number after ear vein injections, and may explain why some injections fail to produce lesions. There is probably some individual variation in elimination, but four other rabbits given 1 cc. and one other rabbit given 2 cc. failed to show any colonies, so the figures given above may be taken as fairly typical.

When bacilli appear, the first plates show the largest number of colonies; as the capillary and rubber tube hold about 0.5 cc. it is evident that the bacilli begin to appear in the bile very quickly and can be demonstrated in collected bile after 2 or 3 minutes. If a large enough dose is given, bacilli will therefore appear in the bile, but they may not be able to multiply and produce a lesion as will appear later when the antiseptic action of rabbit bile is considered.

Relation of the Place of Injection to the Number of Bacilli Appearing in the Bile.—Further evidence along this line is furnished by injection into the mesenteric veins. If gall-bladder infections are due to bacilli in the bile it should be possible to produce more infections by mesenteric than by ear vein injections and more bacilli should appear in the bile in the former instance. Such is the case; after intravenous injection into mesenteric veins seven out of eleven rabbits, or 63 per cent, had gall-bladder lesions at the end of a week, while of twelve controls given the same dose into an ear vein, only five, or 41 per cent, showed a lesion. The number of bacilli appearing in the bile after mesenteric vein injection was larger than after ear vein injection, although there was more variation than in the ear vein injection experiments given above. 1 cc. of a 24 hour broth culture injected into the portal system resulted in thirty-one colonies in the plated bile as compared with none in the control experiment, but in a second experiment no colonies appeared. Similarly, in the case of cholera vibrios, 1 cc. produced twenty-eight colonies in one instance and none in the control, but in a second experiment no colonies appeared. In general, however, on the basis of considerable experience with the two methods, I can state that more organisms appear in the bile after mesenteric vein injection than after ear vein injection. The evident bearing of this fact on production of gall-bladder lesions in cholera and dysentery will be discussed later.

Relation of Immune Animals to the Number of Bacilli Appearing in the Bile.—Evidence is also furnished by the observation of Dr. K. F. Meyer, soon to be published, that immune animals show a somewhat higher percentage of infections of the gall-bladder than normal animals. This condition has been investigated by the same technique mentioned above with results shown in Table II. Meyer immunized the animal with three intravenous injections. Its agglutination was strong at 1: 10,000. The test dose was 1 cc. of a 24 hour broth culture with one control not immunized.

TABLE II.

Number of Colonies in Plated Bile in an Immunized Animal and in a Normal Animal.

Bile.	Normal animal.	Immunized animal, agglutination, 1:10,000.
cc.		
0.5	—	—
0.5	—	50
0.5	—	90
0.5	—	45
0.5	—	11
1.0	—	20
1.0	—	—
1.0	—	1
1.0	—	—
1.0	—	1
Total	0	218
Time of collection in min.	50	50

In this experiment the bacilli in the immunized animal came through in large numbers, while the control showed none. In two other vaccinated animals with an agglutination of only 1: 6,000, only a few colonies appeared. These results can be best explained in accordance with the recent work of Bull (13) by the rapid agglutination *in vivo*, the deposition of bacilli in the liver, and corresponding elimination. In other words, gall-bladder infections in vaccinated animals are not necessarily an index of lack of immunity, but may, in part, be an indication of a rich amount of immune bodies in the blood. Greig (16) has reported the occurrence of gall-bladder lesions in rabbits highly immunized with cholera-like vibrios. These results confirm my previous contention (17) that the gall-bladder lesion in the rabbit cannot be used to test general immunity from vaccination, and conclusions based on gall-bladder infections in immunized animals must be revised with this fact in mind.

The first condition necessary to confirm the descending infection theory, namely, the presence of bacilli in the bile, is fulfilled by the work stated above; more bacilli appear with larger doses, more bacilli appear with mesenteric vein injection than with ear vein in-

jection, and more bacilli appear in immunized animals. In regard to the theory of infection through the gall-bladder wall, on a critical examination of the work of Chiarolanza who tied the cystic duct and secured infection after intravenous injection, it is found that his protocols state that the bile is bloody even in animals examined in 24 hours, and he obtained 100 per cent of infections of the gall-bladder with the cystic duct tied and only 74 per cent of infections among normal animals.

Chiarolanza apparently assumed that the bloody appearance of the bile was due entirely to infection. As a control on this experiment, I tied the cystic duct of two rabbits but gave no injection; the next day the animals were chloroformed and the bile in the gall-bladder was examined. It had a reddish color and contained desquamated epithelium. It appears, therefore, that in Chiarolanza's work the bile was simply a diluted blood culture, as in tying the cystic duct the cystic artery is also tied and hemorrhagic infarction occurs from collateral circulation. As has been mentioned above, Doerr performed the same experiment of tying the cystic duct, but waited several days before giving an intravenous injection, and under these conditions no infection occurred. In other words, the bladder wall had recovered to such an extent that no blood and consequently no bacilli entered the gall-bladder. Doerr's experiments are therefore much more conclusive than Chiarolanza's and they support the descending infection theory.

The mechanism of infection in paratyphoid infections is in all probability the same as that in typhoid. Carriers are well known in paratyphoid A (18) and B (19, 20) infections, and acute and chronic cholecystitis has been described in both cases. In the rabbit Doerr (12) has reported persistent gall-bladder lesions after intravenous injections of both organisms. In four of fifteen animals I have obtained gall-bladder lesions with intravenous injections of a recently isolated paratyphoid A bacillus.

Gall-Bladder Infection in Cholera and Dysentery as a Result of Portal Septicemia.—As has been shown above, the evidence points to descending infection of the bile from the liver as the cause of gall-bladder infection in a septicemia like typhoid.

In cholera, Schöbl, favors the ascending route from the duodenum and cites the vomiting of cholera vibrios as evidence that they may be present in the duodenum and stomach. Greig (2) refers to a lymphatic infection, but it is not clear how the lymph reaches the gall-bladder from the intestines. In a later article (21) Greig apparently also accepts the direct infection of the gall-bladder wall. Kulescha favors the theory of descending infection on account of foci of infection found in the liver. While cholera is not usually classed as a septicemia and cholera organisms have been recovered from the blood rarely, Greig (2) reports their presence in the lungs, and they have been found in the urine a number of times (22).

These facts point to an occasional invasion of the blood and it seems likely that if blood cultures could be made from the mesenteric veins positive results would be more frequently obtained. Experimental evidence favors this mechanism of gall-bladder infection as it has been shown above that more organisms appear in the bile after mesenteric vein injection than after ear vein injection. The gradual development of immunity would also favor the deposition of organisms in the liver and their increased elimination by the bile.

In dysentery, blood cultures are occasionally successful (4) and dysentery bacilli have been found in various organs, including the gall-bladder, at autopsy. In cholera and dysentery it seems likely, therefore, that gall-bladder infections are due to a portal or general septicemia with elimination of organisms in the bile as in typhoid. The occurrence of liver abscess in amebic dysentery is suggestive along the same line.

In experimental animals persistent gall-bladder lesions have apparently not been produced by intravenous injections of cholera vibrios. A considerable amount of work has been done with animals by Greig (21), Cano (22), Schöbl (23), Baroni and Ceaparu (24), and others, but in each case in which the vibrios have been found in the bile after intravenous injection the animals died or were killed in a few hours. In cases of survival after injection, Schöbl found no lesions in guinea pigs, and Greig found no lesions in one rabbit.

A possible explanation of the failure in guinea pigs will appear later. In three rabbits given ear vein injections of 1 cc. of a 24 hour broth culture, I found no lesions and no vibrios in the bile at the end of 1 week. In three rabbits given the same dose by a mesenteric vein, one animal had bloody bile, but no vibrios were found in it. Further work on the rabbit with more careful dosage is indicated.

Direct inoculation of the gall-bladder of the guinea pig was uniformly successful in Schöbl's hands, and in the rabbit I have produced three persistent lesions in three trials.

With dysentery bacilli less work has been done. Doerr (12) found the Flexner type in the bile 24 hours after intravenous injection in the rabbit, but not after 4 and 14 days. With the Y type I have produced three lesions after four trials by mesenteric vein injection and one after three trials by ear vein injection. By direct inoculation of the Flexner type, out of five trials, five lesions were present at the end of a week. Further experimental work with intravenous injections is needed. Cholera vibrios and dysentery bacilli may be less able to produce gall-bladder lesions in the rabbit than the organisms of the typhoid and colon groups. The clinical percentage of chronic carriers in cholera and dysentery is apparently less than that in the typhoid group; but this may in part be due to a small percentage of cases with invasion of the blood.

Antiseptic Properties of Rabbit Bile.

The second condition to be determined in testing our working hypothesis, namely, multiplication of the organisms in the bile, evidently depends on the qualities of bile as a culture medium. It seems to be generally assumed that ox bile is a standard of bile and that bile is an excellent culture medium for the organisms of the typhoid group. As a matter of fact both these conceptions are more or less incorrect. There is no standard bile, as bile from different animals differs in its action on microorganisms. Even ox bile and human bile may have a distinctly bactericidal action on the typhoid bacillus, as has been shown by Corrado (25), Talma (26), Pies (27), Fornet (28), Meyerstein (29), and others. It is only by the addition of organic matter such as blood or pus that bile regularly becomes a good culture medium. A suggestive recent finding in this connection is that the lactose bile is inferior to the plain lactose broth for the detection of colon bacilli in water (30, 31).

In the following work the difference between the bile in the gall-bladder and the bile directly from the liver has not been considered, but this may be of some importance as the former is known to be richer in solids and, as Okada (32) has shown, may be less alkaline.

In the case of the rabbit and guinea pig the antiseptic action is strongly marked, as may be seen in Tables III and IV. One loopful of a broth culture of different organisms was put in 1 cc. of different kinds of bile. One loopful was plated immediately and one loopful was plated after 24 hours.

TABLE III.

Antiseptic Action of Bile.

1 loop plated immediately.						
Bile.	Typhoid.	Para-A.	Para-B.	Cholera.	Dysentery (Flexner).	Colon.
Ox	240	320	160	10	160	640
Human	1,000	1,600	1,600	50	400	1,600
Rabbit	400	500	800	40	500	In.*
Guinea pig	400	400	500	5	640	800

* In. indicates innumerable.

TABLE IV.

Antiseptic Action of Bile.

1 loop plated after 24 hours' incubation.						
Bile.	Typhoid.	Para-A.	Para-B.	Cholera.	Dysentery (Flexner).	Colon.
Ox	In.	In.	In.	In.	In.	In.
Human	"	"	"	"	"	"
Rabbit	—	—	—	—	—	—
Guinea pig	—	—	—	—	—	—

This experiment shows that typhoid, cholera, and dysentery organisms grow well in human or ox bile, but are killed after 24 hours in guinea pig and rabbit bile. All specimens of guinea pig bile killed all the organisms tested. In the rabbit there was some variation especially in regard to cholera vibrios; human and ox bile also vary in effect, but the above result is typical of a certain number of specimens. A fairly long exposure is necessary as there is not evidence of antiseptic action at the end of 1 hour. These facts are plainly of considerable importance in many ways. They may explain failure of infection even with doses large enough to insure the passage of

bacilli through the bile. They also furnish a concrete instance of a difference between an experimental animal and man. As a side issue an example may be cited of failure to appreciate this difference. Greig (21) in endeavoring to find some means of preventing cholera carriers, gave rabbits urotropin and tested their bile for vibricidal properties. As a control he used ox bile and while the vibrios grew well in ox bile they failed to grow in the treated rabbit bile. Greig notes the unusual fact that urotropin seemed to be active in an alkaline medium. It is clear from the above results that the vibrios might not have grown in a control of normal rabbit bile.

The reaction of bile from different species varies as much as does the color, and the reaction seems to be of considerable importance in explaining its action on microorganisms. Table V shows the reaction to phenolphthalein and lacmoid; a satisfactory, simple method (32) of titrating the reaction of all specimens of bile has not been found on account of the difference in color. Phenolphthalein is a good indicator for rabbit and guinea pig bile, but it is difficult to use on human and ox bile. With lacmoid the end-point is also difficult to determine exactly.

TABLE V.

Reaction of Bile.

Bile.	Color.	Phenolphthalein.	Lacmoid
Ox	Dull brown.	Acid.	2*
Human	Golden "	"	Neutral.
Rabbit	Bright green.	Neutral.	3–6
Guinea pig	" yellow.	1 per cent alkaline.	8

* The figures for lacmoid show the number of cc. of N/1 acid required to neutralize 100 cc.

These analyses taken together with the bactericidal tests given above suggest that the alkalinity is responsible for the antiseptic action; guinea pig bile, for example, is 2 per cent more alkaline than usual culture media. If guinea pig or rabbit bile is neutralized by hydrochloric acid or sulphuric acid, the antiseptic properties disappear, as is seen in Tables VI and VII, in which the same technique was employed.

TABLE VI.

Antiseptic Action of Fresh Bile.

	Rabbit.		Guinea pig.	
	1	2	1	2
	1 loop plated immediately.	1 loop plated after 24 hours.	1 loop plated immediately.	1 loop plated after 24 hours.
Typhoid	50	—	400	—
Para-A	240	—	400	—
Cholera	10	1,600	5	—
Colon	800	—	640	—
Dysentery	480	—	800	—

TABLE VII.

Absence of Antiseptic Action of Bile When Neutralized.

	Rabbit.		Guinea pig.	
	1	2	1	2
	1 loop plated immediately.	1 loop plated after 24 hours.	1 loop plated immediately.	1 loop plated after 24 hours.
Typhoid	200	In.	800	In.
Para-A	200	"	500	"
Cholera	75	"	15	"
Colon	750	"	400	"
Dysentery	480	"	480	100

The alkalinity of the bile may not be the only factor concerned in its inhibitory action, but it must be an important factor in view of these results. It is instructive to note that of all the organisms, the only one which grew in fresh alkaline bile was the cholera vibrio which is known to grow best in an alkaline culture medium.

Prophylaxis.

These facts naturally suggest that an alkaline therapy might be of value in preventing or curing carriers. The reaction of rabbit bile can be influenced by injections of alkalies. A common duct fistula was made and 20 cc. of a 5 per cent solution of sodium bicarbonate, or 1 gm., were given intravenously and the reaction of the bile was

tested at different times. At the end of an hour the reaction was nearly twice as alkaline as at first and then it fell again.

In order to make a preliminary test of alkaline therapy in preventing gall-bladder infections, four animals were given 1 gm. of sodium bicarbonate in 5 per cent solution, intravenously, and after 1 hour they, with four controls, were given 2 cc. of a suspension of typhoid bacilli, equal to one 24 hour agar slant. At the end of a week they were chloroformed and examined; the controls showed 50 per cent of lesions, the treated animals showed 25 per cent of lesions. In other words, some prophylactic effect was apparently demonstrable. Further work is necessary but the practical suggestion for the trial of alkalies in the prevention and treatment of carriers is obvious.

It is known that gall-bladder infections in the rabbit tend to spontaneous cure, and further work on this subject should be done with a view to determine the part played by the bile in this process.

DISCUSSION.

The results of the experiments recorded above support the theory of descending infection of the gall-bladder through the bile from the liver. Infection of the gall-bladder wall cannot be absolutely ruled out and probably occurs at times, but the bile-ducts seem to be the regular avenue of infection. This conclusion suggests that prophylactic measures and possibly curative measures should be directed toward the bile rather than toward the blood stream and tissues. Vaccination, for example, appears to have little effect in the prevention and cure of experimental or clinical lesions and in fact may favor the production of lesions by increased elimination of organisms in the bile. A great deal of experimental work has been done with various drugs and synthetic substances, but the subject of the natural defences of the bile and the possibility of increasing them has been neglected. Human bile must have some antiseptic action, because, in any septicemia, some microorganisms undoubtedly pass through the bile-ducts and gall-bladder, but in only a comparatively few cases do they produce a definite cholecystitis. *In vitro*, some specimens of human bile are bactericidal or inhibitive, while others are not. Experiments into the nature of this inhibition with a view to increasing it have been done with the rabbit.

Rabbit bile is more alkaline than human bile and is more antiseptic for all the organisms considered in this paper, except in some instances for cholera vibrios. Rabbit bile has some effect in preventing lesions, because doses large enough to insure the passage of organisms through the bile and large enough to make an animal sick or even kill it often show no organisms on culture of the bile. Gall-bladder lesions in the rabbit are probably due, first, to a sufficient dose to insure a considerable number of microorganisms in the bile; second, to a low margin of the inhibitory action in the bile or to an increased resistance on the part of the strain employed; and third, to an injury of the liver cells or mucous membrane of the gall-passages and bladder with desquamation of cells and oozing of serum. Pies (27) showed that *in vitro* the inhibitory action of the bile could be abolished by the addition of serum or pus. In the direct inoculation of the gall-bladder this factor probably is important in the almost invariable success of the method. In man the production of gall-bladder lesions probably depends on the same factors as in the rabbit. The difference between rabbit and human bile is only a question of degree, and valuable lessons may be learned from the rabbit, especially in regard to the cause of failure of intravenous injections to produce 100 per cent of lesions.

It is a debatable question whether a patient suffering from an acute infection should be subjected to additional treatment in order to prevent him from the possibility of being a danger to others, provided he recovers, but if a mild therapy were of any value it would be justified. In cholera, for example, alkaline therapy is of value in preventing uremia, which is the greatest danger to the individual, next to collapse (33). If this treatment also helped to keep the bile clean, this fact should be kept in mind. However, as the cholera vibrio grows best in an alkaline medium, this line of treatment may favor cholecystitis. These questions are subjects for further investigation. When the individual recovers from an acute attack, the best practice requires that he shall not be discharged as cured until the carrier question is considered. Aside from the examination of the stool, the use of Einhorn's duodenal tube (34) seems to be a valuable aid in determining the condition of the bile.

SUMMARY.

1. The theory of the production of gall-bladder lesions in typhoid, by descending infection of the bile from the liver receives support from investigations with the common duct fistula method in the rabbit.

More bacilli appear in the bile with increased doses and more gall-bladder infections are obtained by increased doses.

More bacilli appear in the bile after mesenteric vein injection than after ear vein injection and more lesions result under the first condition.

More bacilli appear in the bile after injection of the same dose in immunized animals than in normal animals and more lesions also result in immunized animals.

In cholera and dysentery the same mechanism is suggested with the additional factor of a portal system septicemia.

2. After the appearance of microorganisms in rabbit bile, their fate is apparently largely determined by the antiseptic properties of the bile.

100 per cent infections cannot be secured by intravenous doses large enough to insure the presence of microorganisms in the bile.

Rabbit bile *in vitro* may be antiseptic to the microorganisms considered.

The antiseptic action is largely due to its alkalinity.

It is apparently possible to protect the rabbit to some degree against gall-bladder infection by a previous injection of sodium bicarbonate.

3. Alkaline therapy is suggested in the prevention and cure of gall-bladder carriers.

BIBLIOGRAPHY.

1. Kulescha, G. S., *Klin. Jahrb.*, 1911, xxiv, 137; *Centr. Bakteriol., 1te Abt., Orig.*, 1909, l, 417.
2. Greig, E. D. W., *Indian J. Med. Research*, 1913–14, i, 44, 59; 1914–15, ii, 1, 28.
3. Schöbl, O., *Philippine J. Sc., B*, 1915, x, 11.
4. Ghon, A., and Roman, B., *Wien. klin. Woch.*, 1915, xxviii, 579.
5. Koch, J., *Z. Hyg. u. Infectionskrankh.*, 1909, lxii, 1.
6. Chiarolanza, R., *Z. Hyg. u. Infectionskrankh*, 1909, lxii, 11.
7. Lange and Roos, *Arb. k. Gsndhtsamte.*, 1915, l, 57.

8. Anton, B., and Fütterer, G., *Münch. med. Woch.*, 1888, xxxv, 315.
9. Fütterer, G., *Medicine*, 1895, i, 279.
10. Fütterer, *Berl. klin. Woch.*, 1899, xxxvi, 58.
11. Biedl, A., and Kraus, R., *Centr. inn. Med.*, 1896, xvii, 737.
12. Doerr, R., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 624.
13. Bull, C. G., *J. Exp. Med.*, 1915, xxii, 475.
14. Blachstein, A. G., *Bull. Johns Hopkins Hosp.*, 1891, ii, 96.
15. Welch, W. H., *Bull. Johns Hopkins Hosp.*, 1891, ii, 121.
16. Greig, *Indian J. Med. Research*, 1916, iii, 259.
17. Nichols, H. J., *J. Exp. Med.*, 1914, xx, 573.
18. Kayser, H., *Deutsch. med. Woch.*, 1904, xxx, 1803.
19. Forster, J., and Kayser, H., *Münch. med. Woch.*, 1905, lii, 1473.
20. Irons, E. E., and Jordan, E. O., *J. Infect. Dis.*, 1915, xvii, 234.
21. Greig, *Indian J. Med. Research*, 1914–15, ii, 907.
22. Cano, U., *Centr. Bakteriol., 1te Abt., Orig.*, 1914, lxxii, 124, 125.
23. Schöbl, *J. Infect. Dis.*, 1916, xviii, 307.
24. Baroni, Y., and Ceaparu, V., *Compt. rend. Soc. biol.*, 1912, lxxii, 894.
25. Corrado, B., *Centr. Bakteriol.*, 1892, xi, 696.
26. Talma, S., *Z. klin. Med.*, 1901, xlii, 354.
27. Pies, W., *Arch. Hyg.*, 1907, lxii, 107.
28. Fornet, W., *Arch. Hyg.*, 1907, lx, 134.
29. Meyerstein, W., *Centr. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 434.
30. Cumming, H. S., *Bull. Hyg. Lab., U. S. P. H.*, 1916, No. 104, 105.
31. Obst, M. M., *J. Bacteriol.*, 1916, i, 73.
32. Okada, S., *J. Physiol.*, 1915, l, 114.
33. Sellards, A. W., *Philippine J. Sc., B*, 1910, v, 363.
34. Einhorn, M., *J. Am. Med. Assn.*, 1916, lxvi, 1908.

STUDIES ON THE BLOOD PROTEINS.

I. The Serum Globulins in Bacterial Infection and Immunity.

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INTRODUCTION.

For a number of years much study has been devoted to the origin and the chemical nature of the antibodies which may develop within the organism during the course of an infection, or which may be elaborated within it by the various methods of immunization. The efforts to establish the chemical identity of antibodies have naturally been centered about a study of the possible relationship subsisting between the proteins of the blood and the immune bodies demonstrable in it by various serologic tests. A great stimulus to these investigations has come from the discovery of new methods of separating and of chemically identifying the different fractions which go to make up the blood proteins. Of these additions to our knowledge the method, introduced by the Hofmeister school, of separating the various protein constituents by fractional precipitation with different salts has, perhaps, produced the most far reaching results.

For some time it has been a well established fact that diphtheria antitoxin, for instance, is precipitable from serum by any precipitants which throw down the globulins. The early observations of Brodie (1), Seng (2), and Hiss and Atkinson (3) have been confirmed and extended by a number of later workers (4).

Considerable work has been done also to establish the chemical nature of bacterial antibodies. Probably one of the earliest contributions to this subject was made by Pfeiffer and Proskauer (5), who separated cholera immune serum into its globulin and albumin fractions, and showed that the cholera immune bodies which give rise to Pfeiffer's phenomenon are present only in the globulin fraction. This important study was later amplified by the classical experiments of Pick (6), who demonstrated conclusively that cholera and typhoid agglutinins also occur in one or another of the globulin fractions depending upon the species

of animal employed for immunization and the nature of the antibody studied. To these observations should be added those of Rodhain (7) and of Moll (8). According to the work of Rodhain, the immune bodies of antistreptococcus serum occur in the euglobulin fraction; and according to Moll, the development of precipitins following immunization with a foreign protein is also associated with a rise in the serum globulins of the immunized animal.

The above observations have been variously interpreted by different investigators. Whereas some workers strongly incline to the view that the antibodies in question are a form of blood globulin, others entertain the possibility that the antibody, by analogy with bacterial poisons, enzymes, and similar bodies, is mechanically carried down by the precipitate of globulin.

We believed that the only satisfactory method of procuring reliable data on the globulin-antibody problem was to make quantitative estimations of the immune bodies and of the blood proteins, not at random periods during the experiment, but at frequent and well timed intervals during the process of immunization. In this way alone is it possible to determine whether an increase in the antibodies and in the globulins parallels one another, or whether either the globulin content or the concentration of immune bodies may increase independently of one another.

Methods.

Healthy Belgian hares kept under constant conditions of diet and activity were used for all the experiments. The animals were fed once daily, the diet consisting of alfalfa hay, toasted bread, oats, and at times small amounts of cabbage. All were allowed a liberal amount of water.

Obtaining Blood.—Specimens of blood, varying in amounts from 5 to 7 cc. were obtained from fresh incisions made in the ear vein. In all instances the use of local applications of xylol or other substances that might cause stasis was avoided. At times the presence of a low blood pressure made bleeding difficult, but in such cases a free flow of blood was obtained by suspending the rabbit by its hind legs. The blood obtained in small sterile tubes was immediately centrifugalized in order to obtain a clear serum. Separation of the serum from the clot was effected as soon as possible, for as will be shown later, serum allowed to remain in contact with the clot is not suitable for accurate determinations of the proteins. The specimens before use were kept in sterile, stoppered vials in the refrigerator.

Tests for Agglutination.—The antigen used for the agglutination tests consisted of a 24 hour carbolized or formalinized Liebig's or rabbit broth cul-

ture, which had been properly controlled by tests with immune sera of known antibody content. To a series of tubes containing the clear, untreated serum in amounts ranging from 0.1 cc. to 0.00005 cc. was added 1 cc. of antigen. After incubating the mixtures for 2 hours at 37° C. or at room temperature for 12 hours readings were made. The final readings were always made atter the lapse of 12 or 14 hours. The highest dilution of the serum in which complete agglutination occurred was taken to represent the agglutination titer of that serum, and only these readings are recorded in the tables.

Tests for Complement Fixation. Antigens.—Cultures of the various organisms grown upon lemco broth[1] for 18 to 20 hours[2] were found to be most suitable for this purpose. They were killed by heating for ½ hour at 60°C., and preserved with 0.5 per cent carbolic acid and 1 per cent glycerol. When kept in the refrigerator and protected from the light such antigens may be ready for use after a period of 2 months. Some, however, may become anticomplementary after a period of 4 weeks, and these must be discarded.

The dose of antigen employed was four times the antigenic unit as determined by preliminary titrations of the antigen with a standard amount of immune serum; either 0.1 or 0.2 cc. This dose was at least one-quarter to one-fifth of the anticomplementary unit as determined by repeated titrations. The range of the specific antigenic properties of each antigen was further controlled by tests with sera of known antibody content.

Sera.—Dilutions of inactivated serum (62°C. for 30 minutes) in descending doses from 0.2 to 0.003 cc. were used. In selecting such dilutions it frequently occurred that the gradations were not well chosen. This made it difficult to express the results absolutely in terms of the highest dilution of serum which gave definite fixation. For this reason the signs >< are employed. Thus the notation > 0.005 indicates that complete fixation of the complement would probably have occurred in a serum dilution of 0.004 or 0.003 cc., since in a dilution of 0.002 cc. of the serum only 50 per cent fixation was obtained. In the tables only those dilutions of the serum are recorded which with the proper dose of the antigen caused a complete fixation of the complement.

Hemolytic System.—The anti-sheep hemolytic system was used. Complement was furnished by the pooled sera of several guinea pigs, and was employed in a dosage of 0.05 cc. of a dilution of 1 to 4 in salt solution. The red corpuscles were used in 1 per cent suspension. The hemolytic unit was determined by a preliminary test using 0.05 cc. of complement and 0.5 cc. of a 1 per cent suspension of fresh sheep cells. In the titrations of the antigens as well as for the actual complement fixation tests two hemolytic units (about 0.2 to 0.1 cc. of a dilution of 1 to 100 in saline solution) were employed.

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[1] Eyre, J. W. H., The Elements of Bacteriological Technique; a Laboratory Guide, Philadelphia and London, 2nd edition, 1913, 163.

[2] Cultures less than 18 hours old are frequently inactive, and those older than 24 hours may be anticomplementary in doses of 0.5 cc.

Technique.—Preliminary tests were first made to rule out any anticomplementary activity of the different antigens used. These were carried out as follows: To each of a series of tubes containing decreasing doses of antigen diluted with 1.5 cc. of isotonic salt solution was added 0.05 cc. of complement in a dilution of 1 to 4. The tubes were then incubated at 37° C. either in a water bath for ½ hour or in an incubator for 1 hour. To each tube was then added a previously prepared mixture of two units of hemolysin and 0.5 cc. of the corpuscle suspension. After mixing and incubating for 1 or 2 hours, sedimentation of the red cells was hastened by placing the tubes in the refrigerator so as to make the readings more precise.

After determining the dosage of antigen to be used in the final test, the latter is carried out in the following manner: To each of a series of tubes containing the inactivated serum in descending doses is added 0.05 cc. of complement in 1.5 cc. of saline solution followed by the proper dose of antigen. After incubation for a period of ½ to 1 hour, the sensitized corpuscle suspension is added to each tube in the dosage already given, the mixture reincubated for 1 or 2 hours, and the readings are made as already indicated.

The customary controls for the serum, antigen, and hemolytic system were employed.

Non-specific fixations of the complement by rabbit sera, so frequently observed with bacterial antigens, must, of course, be kept in mind. Such a possibility, however, was ruled out by careful preliminary tests of the serum before immunization with numerous antigens, a procedure recommended by Kolmer and Trist (9). For various reasons it was not possible to select only those rabbits whose sera showed at the outset negative reactions. But we consider that this is unnecessary in serial studies, inasmuch as non-specific fixation does not interfere with specific deviations of the complement due to the presence of immune bodies.

Tests for Antistaphylolysin.—The staphylolysin used in the tests was prepared from a recently isolated strain of *Staphylococcus aureus* grown on a medium having an ionization equal to the value $P_H^+ = 7.7$. Two units of this hemotoxin suspended in isotonic salt solution were added to a series of test-tubes containing the inactivated serum in descending doses. The total volume was now made up to 2 cc., and the mixtures were incubated for 15 minutes in a water bath at 37° C. To each tube was then added 0.05 cc. of a suspension of red blood corpuscles prepared by washing the cells and adding an amount of saline solution equal to the original blood volume. The mixtures were now incubated for 1 hour. The readings were made at the end of 2 hours and again after 12 hours, the figures in the tables indicating the dilutions of serum in which the lytic activity of the staphylohemotoxin was completely inhibited.

Quantitation of Serum Proteins.—Those who have heretofore studied the problem of the relationship of the blood proteins to immunity have in the main obtained their data by precipitation of the globulins and the subsequent Kjeldahl determinations of the nitrogen contained in the precipitate and in the coagu-

lated proteins of the whole serum. Other workers have resorted to the less accurate method of weighing the precipitates. Neither of these procedures is applicable to a systematic study requiring frequent observations upon small animals because of the need of large quantities of blood and the time-consuming character of these procedures.

All the determinations of the albumin, globulin, and non-protein constituents in the blood of the animals experimented upon by us were made by the micro-refractometric method of Robertson (10). As the author has shown in numerous publications, the results obtained by this method are in accord with those obtained by the older methods, and the procedure possesses the important advantages of being less laborious and of being applicable to small quantities of serum.

In brief the method is as follows:[3] Blood is collected in centrifuge tubes, allowed to clot, and centrifugalized to obtain a clear serum. The blood should be obtained before a feeding, since lipemic sera are read with more difficulty. Furthermore, the serum should not be allowed to remain in contact with the clot for any length of time, nor should bacterial contamination be permitted, especially if it is desired to keep the serum for 24 or 48 hours before analyzing it.

By actual experiment we became assured that serum, and more particularly immune serum, may dissolve out substances from the clot which may considerably alter its refractive index. It has been found, for instance, that after 48 hours a sterile immune serum[4] kept in contact with the clot at low temperatures already showed a reduction in the protein quotient. On the other hand, the clear serum, immediately separated from the clot and kept under similar conditions, showed no marked changes after a period of 72 hours. A little over 1.5 cc. of serum is sufficient for the determination of the four fractions.

The tests are carried out in glass tubes having an inside diameter of about 5 mm. and walls about 1 mm. thick. These are sealed at one end.

For the determination of the albumin and globulin, 0.5 cc. of a saturated solution of ammonium sulphate is introduced with the aid of a graduated pipette into one of the tubes, about 10 cm. in length. With the same pipette, which has been cleaned by washing with water, alcohol, and ether, and dried by passing through it a stream of cold air, is added the same amount of clear serum. For purposes of mixing, a piece of silver wire is dropped into the tube, a stopper—consisting of a piece of sealed glass tubing inserted into a piece of rubber tubing—is affixed and the mixture of serum and sulphate is now shaken thoroughly. The precipitate of globulin is sedimented by centrifugalization, the clear fluid is diluted with a graduated pipette to one-half and its refractive index determined.

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[3] For details concerning the various steps in the method, and for a discussion of the reasons for them and of the manner of calculating the results, reference should be made to Robertson (10).

[4] This immune serum exhibited a high antibody content. On May 8 the serum agglutinated in a dilution of 1:4,000, and fixed the complement in a dilution of 0.001 cc. of serum.

This reading corrected for the ammonium sulphate gives the total albumin plus the non-protein.

The non-protein value is determined by mixing in a similar glass tube 0.5 or 1 cc. of the clear serum with an equal volume of 0.04 N acetic acid solution. A short piece of silver wire is now dropped into the tube, the upper end is sealed off in the flame, the mixture shaken, and coagulated by placing the tubes in a beaker of water heated to boiling for several minutes. This precipitate also is sedimented by centrifugalizing and the refractive index of the clear supernatant fluid is determined.

Lastly the refractive index of the whole serum is determined. From this reading the refractive index of the total globulin is obtained by subtracting the refractive reading of the albumin from that of the whole serum after deducting the value of the non-proteins.

The readings were made with a Pulfrich refractometer, and the calculations of the percentages of the various constituents were carried out in the manner presented in detail by Robertson. The results are expressed not only in percentage but also in the per cent of total protein. For purposes of graphic presentation, it was thought well to express the ratio of albumin to globulin in the form of a quotient. This was obtained by dividing the percentage of albumin by that of globulin. Thus a fall in the quotient would indicate a rise in the blood globulins, and *vice versa.*

From a large series of determinations, numbering several hundred, we have become convinced of the accuracy of this method, provided the sources of error are understood and proper care is exercised in the manipulations. The method is especially recommended on account of the rapidity with which the determinations can be made and the small quantities of serum required.

EXPERIMENTAL.

Observations on the serum proteins were made in normal, infected, immunized, and hyperimmunized animals. With the exception of some of the infected animals, a parallel study was made also of the degree of immunity present during different periods of the experiment. As a typical example of an acute infection, staphylococcus pyemia was chosen. Infections with the tubercle bacillus and with sporothrix were selected as types of chronic infections.

For purposes of immunization living and killed cultures of *Bacillus typhosus* and *Bacillus dysenteriæ* (Shiga) and *Staphylococcus pyogenes aureus* were used. In addition to the classical method of immunization, a study was made also of the effect of massive inoculations in normal and immune animals upon the albumin-globulin ratio and

upon antibody formation. To these were added several observations upon the changes produced in the serum proteins by the inoculation of bacterial endotoxins and inflammatory irritants.

Serum Proteins of Normal Rabbits.

Observations on the serum proteins were made on a dozen normal rabbits kept under constant conditions of diet and activity. Notwithstanding the constancy of the conditions, it is apparent that individual animals may show considerable variations in the percentage of the serum proteins. Thus the total proteins may vary from 5 to 7 per cent. The albumin fraction may show fluctuations from 3.1 to 5.5 per cent, and the globulins from 0.8 to 2.7 per cent. But the averages of all the readings yield values which are in fair accord with those of other workers (11), especially in so far as the albumin-globulin ratio is concerned. Such fluctuations as have been observed in the protein quotient in normal animals are small in comparison with the marked diminution in the quotient which has been noted in the pathological conditions studied. Whereas the quotient in normal animals averaged 3.5, and in most instances did not fall below 1.5, infected and immunized animals have at one period or another shown a quotient below 1.0. Furthermore, it is essential in all these experiments to determine the percentages of the serum proteins existing in the normal animal before proceeding to determine the variations which may follow the establishment of a pathological condition.

Infection.

Experiment 1. Infection. Pyemia.—Two rabbits were inoculated intravenously with potato cultures of *Staphylococcus pyogenes aureus.* One of the animals (Rabbit 1) received on February 17 two loopfuls of a culture, and died 8 days later of a bilateral fibrinous pleurisy, and abscesses in the lungs and kidneys. The second animal (Rabbit 2) was inoculated on March 2 with only one loopful of a potato culture. This animal emaciated gradually, losing about 600 gm. in weight, and was killed 13 days later.

Autopsy.—Animal anemic and emaciated. Pleural and peritoneal cavities contain a considerable amount of fluid. Atrophy of mesenteric fat. Slight engorgement of liver and spleen. Abscesses and infarcts in both kidneys. Large abscess in muscles of right hind leg. Fibrinopurulent arthritis ot right coxofemoral joint. Bone marrow gelatinous and deep red in color.

The serum proteins were studied at frequent intervals during the course of the infection, and the results obtained showed clearly the fluctuations which may occur in the blood proteins in a typical infection with *Staphylococcus aureus.* The progress of the infection in both animals studied was accompanied by some antibody response as evidenced in the formation of antistaphylolysins. These developed to an equal degree in both rabbits. In Rabbit 2, however, the changes in the percentages of the various constituents, and especially in the albumin-globulin ratio, were not marked. The striking result of this experiment was the precipitous drop in the protein quotient observed in Rabbit 1, which received a massive inoculation of staphylococci. Of interest, too, is the fact that this rise in the total globulins was associated with a parallel fall in the total albumin.

The loss in weight in both animals was considerable, but it is of interest that this loss occurred more rapidly in Rabbit 1 (3 days), whereas as in Rabbit 2 it was more gradual. The significance of this observation will become more apparent in connection with other experiments.

Experiment 2. Tuberculosis.—On February 17 a rabbit weighing 2,600 gm. was inoculated intravenously with 0.1 mg. of a 72 day culture of bovine tubercle bacillus grown on tuberculin agar. During the progress of the infection, frequent analyses of the serum proteins were made, and the albumin-globulin ratio was determined. These observations are presented in Table I.

The animal emaciated only moderately, and was killed about $2\frac{1}{2}$ months later.

Autopsy.—Extensive pulmonary tuberculosis with cavity formation. Numerous cheesy foci and miliary tubercles. Tuberculosis of mediastinal lymph nodes. Tubercles in spleen and kidneys.

Experiment 3. Infection with Sporotrichum schenckii and beurmani.—A rabbit weighing 1,750 gm. was injected intraperitoneally with 2 cc. of a cream suspension of sporothrix (No. 3725, 1912) grown in 4 per cent glucose broth since October 25, 1915. The animal gained in weight and 10 days later 4 cc. of the same culture were injected. On the 30th day a third and final injection of 6 cc. was given. On March 17 three nodules about the size of large cherries were felt at the site of inoculation.

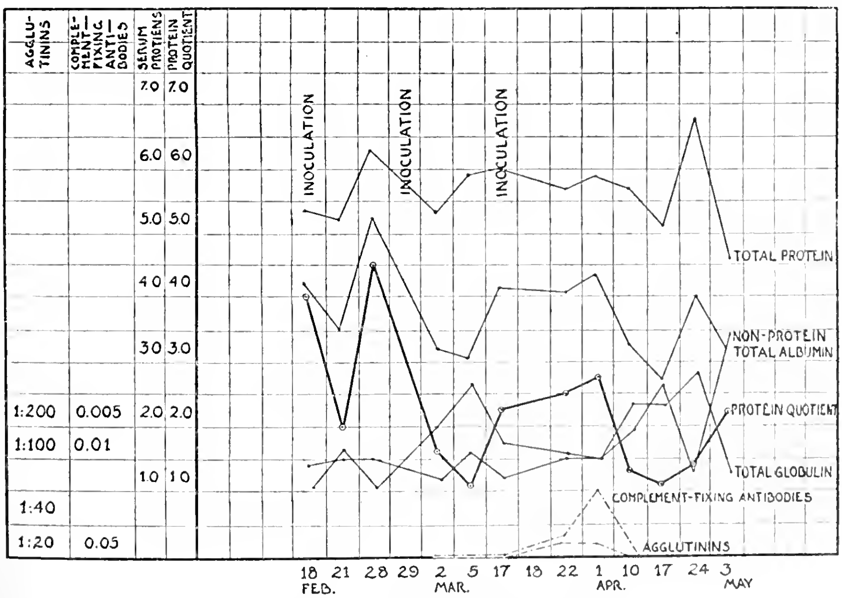
Frequent observations were made upon the agglutinins and complement-fixing antibodies as well as upon the serum proteins (Text-fig. 1). The animal was killed about $2\frac{1}{2}$ months later.

Autopsy.—At the site of the injections there were many pea-sized nodules. These were present both in the subfascial layers and in the abdominal muscles.

TABLE I.

Experiment 2. Chronic Infection. Tuberculosis. Rabbit 3.

Date.	Weight.	Organisms inoculated.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	gm.		per cent	per cent	per cent	per cent	per cent	per cent		
Feb. 17..	2,600	0.1 mg. intravenously.	6.6	5.3	1.3	80	20	1.2	4.0	Bovine strain grown on tuberculin agar, 72 days old.
" 21..	2,400	—	6.0	4.6	1.4	76	24	1.5	3.1	
" 25..	2,625	—	—	—	—	—	—	—	—	
" 28..	2,400	—	6.5	4.7	1.8	72	28	1.6	2.9	
Mar. 9..	2,700	—	6.0	4.2	1.8	70	30	1.4	2.3	
" 17..	2,400	—	6.8	4.6	2.2	68	32	1.4	2.1	
Apr. 1..	2,550	—	7.2	4.4	2.8	61	39	1.5	1.6	
" 10..	2,550	—	6.0	3.1	2.9	51	49	2.6	1.0	
" 17..	2,450	—	5.2	2.1	3.1	40	60	3.5	0.66	
" 24..	2,350	—	6.9	3.6	3.3	52	48	1.7	1.1	
May 3..	2,300	—	5.0	0.5	4.5	10	90	3.5	0.1	Animal killed. Blood for examination obtained from heart.



TEXT-FIG. 1. Infection with sporothrix.

Similar ones were found also in the omentum, between the loops of intestine and between the liver and diaphragm. Histologically, they were found to show the presence of typical sporotrichotic granulation tissue. From one of these nodules a positive culture was obtained.

Both the tubercular and mycotic infections were characterized by a long chronic course of several months associated with only slight wasting. Immediately following the inoculation, in the one instance with the tubercle bacillus and in the other with the sporothrix, each animal showed a slight rise in the total globulins as evidenced by a fall in the protein quotient. But it is of interest that the latter continued at a fairly constant level, and that the fluctuations observed usually followed the intraperitoneal injections. Thus it will be noticed the protein quotient did not show such a precipitous fall in the chronic as in the acute infections.

In the animal infected with sporothrix a slight grade of immunity developed, but neither the agglutinins nor the complement-fixing antibodies ever rose to a high level. Notwithstanding this lack of response, the serum proteins still showed striking fluctuations. The curve of total albumin for the most part paralleled closely the curve of total proteins. The globulins showed a tendency to rise when the albumin curve fell, but this was not the case for all periods of the experiment. Both of these features are well represented by the fluctuations of the protein quotient as shown graphically in the text-figure.

Immunity.

Experiments 1 and 2. Immunization with Bacillus typhosus.—Two healthy rabbits were inoculated with *B. typhosus*. One animal received inoculations of stock strain and the other Army vaccine. Serum samples were obtained before the injection for a study of the agglutinins, complement-fixing antibodies, and serum proteins. Similar observations were made at frequent intervals following the inoculations. The details of Experiment 2, in which Army vaccine was used, are recorded in Table II and Text-fig. 2.

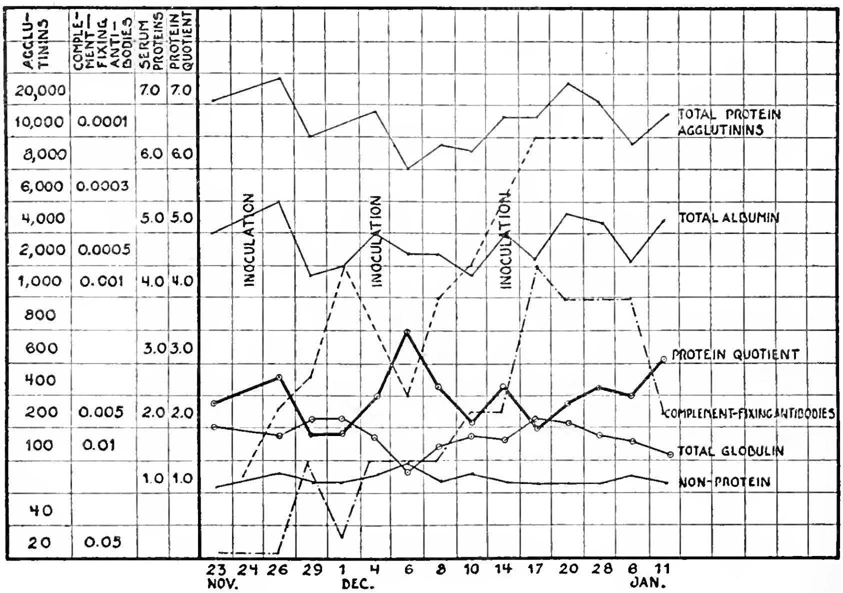
In both of the experiments on typhoid immunization the inoculations were given in such dosage and at such intervals as to develop within the organism a maximum degree of immunity without causing any marked metabolic disorder. Both animals maintained their weight at a normal level throughout the period of immunization, and

TABLE II.

Experiment 2. Typhoid Immunization. Rabbit 4.

Date.	Organisms inoculated.	Antibodies.		Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		Agglutinins.	Complement-fixing antibodies.	per cent	per cent	per cent	per cent	per cent	per cent		
Nov. 23......	—	1:20	0.05	7.0	5.0	2.0	71	29	1.1	2.4	
" 24......	0.5 cc. (250,000,000) intravenously.	1:80	0.05	—	—	—	—	—	—	—	Army vaccine. Weight 3,800 gm.
" 26......	—	1:320	0.05	7.4	5.5	1.9	74	26	1.3	2.8	
" 29......	—	1:500	<0.01	6.5	4.3	2.2	66	34	1.2	1.9	Weight 3,400 gm.
Dec. 1......	—	1:2,000	0.03	6.7	4.5	2.2	67	33	1.2	2.0	
" 4......	—	1:800	0.01	6.9	5.0	1.9	72	28	1.3	2.5	
" 5......	1.0 cc. (500,000,000) intravenously.	—	—	—	—	—	—	—	—	—	
" 6......	—	1:400	<0.01	6.0	4.7	1.3	78	22	1.4	3.5	
" 8......	—	1:1,000	<0.01	6.4	4.7	1.7	73	27	1.2	2.7	
" 10......	—	1:2,000	0.003	6.3	4.4	1.9	69	31	1.3	2.2	
" 14......	—	1:6,000	0.003	6.8	5.0	1.8	73	27	1.2	2.7	Weight 3,800 gm.
" 15......	1.0 cc. (500,000,000) intravenously.	—	—	—	—	—	—	—	—	—	
" 17......	—	1:10,000	<0.0005	6.8	4.6	2.2	67	33	1.2	2.0	
" 20......	—	1:10,000	0.001	7.4	5.3	2.1	71	29	1.2	2.4	
" 28......	—	1:10,000	0.001	7.1	5.2	1.9	73	27	1.1	2.7	Weight 3,500 gm.
Jan. 6......	—	—	0.001	6.4	4.6	1.8	72	28	1.3	2.5	
" 11......	—	—	0.003	6.8	5.2	1.6	76	24	1.1	3.1	

the immunity developed after the third inoculation was of a high grade in each instance. In one of these animals (Rabbit 5), the agglutinins showed a tendency to fall at one period following an intercurrent infection resulting from an abortion. Soon after this reduction in the agglutination titer, there occurred also a definite rise in the serum globulins. The association of the development of a pyemia with a high globulin content has been a frequent observation. Its significance will be discussed in subsequent paragraphs.



TEXT-FIG. 2. Typhoid immunization.

In neither animal was it possible to demonstrate any direct parallelism between the rise in the immune bodies and the fluctuations in the serum globulins. The latter showed a tendency to rise (fall in protein quotient) 24 to 48 hours following an inoculation, and a tendency to return to a normal level in the following several days.

Text-fig. 2 represents these fluctuations graphically for Experiment 2. It will be observed that after a period of about 3 weeks following the initial inoculation the value of the protein quotient showed no tendency to change materially, although the development of immune

bodies had reached its highest point. The total proteins and total albumins showed parallel fluctuations but no definite tendency to rise during the process of immunization.

Experiment 3. Immunization with Dysentery Bacillus (Shiga).—A rabbit weighing 2,450 gm. was inoculated intravenously with increasing doses of living dysentery bacilli (Strain Do, Pasteur Institute, December, 1913) suspended in salt solution. The first inoculation was given on January 27 when 0.01 of a loop (20,000 organisms) was inoculated. The same dose was given 10 days later. 20 days after the first inoculation the animal received ten times this number of organisms. This was increased to 100 times the dose on the 30th day. On the 40th and 50th days, two and one-third and eight loopfuls, respectively, were inoculated. These injections were all well tolerated and were not followed by loss in weight. The degree of antibody response and the change in the serum proteins are recorded in detail in Table III and Text-fig. 3.

8 weeks after the beginning of the experiment, the animal died of exsanguination following prolonged bleeding from the ear artery.

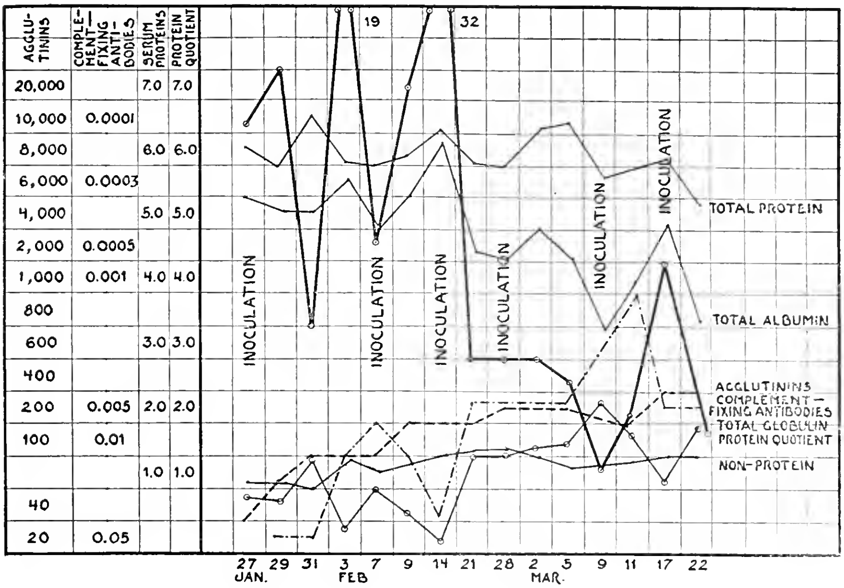
The striking changes in the blood globulins brought about by the inoculation of living dysentery bacilli are well shown in the text-figure. It will be observed that following the first two inoculations both the albumin and globulin curves showed wide fluctuation, and that only after the third inoculation did the globulins show a gradual upward course and the albumin a gradual downward course. During two periods of the experiment (February 3 and 14) the albumin fraction rose to a high level. A similar observation was made upon an animal immunized with living staphylococci. Apart from the explanation that the injection of living organisms may give rise to a marked metabolic disorder, the reasons for such extreme variations in the curve are not clear, unless it is assumed that the active multiplication of bacteria may bear some relation to these fluctuations.

The agglutinins and complement-fixing antibodies rose gradually reaching their highest level during the 5th week. But as a careful analysis of the antibody and globulin curves will disclose, there is a marked fluctuation of the latter curve throughout its entire course. The most striking discrepancy was noted on March 17, when the concentration of antibodies had reached its maximum; whereas the globulin content was beginning to return to its initial level.

TABLE III.

Experiment 3. Immunization with Dysentery Bacillus (Shiga). Rabbit 6.

Date.	Organisms inoculated.	Antibodies. Agglutinins.	Antibodies. Complement-fixing antibodies.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Jan. 27.....	0.01 loop (20,000) intravenously.	0	0.03	6.3	5.5	0.8	87	13	1.2	6.7	Weight 2,450 gm.
" 29.....	—	1:80	0.03	6.0	5.3	0.7	88	12	1.2	7.5	
" 31.....	—	1:100	0.03	6.8	5.3	1.5	78	22	1.0	3.5	Weight 2,100 gm.
Feb. 3.....	—	1:100	0.01	6.1	5.8	0.3	95	5	1.5	19	
" 7.....	0.01 loop intravenously.	1:100	0.005	6.0	5.0	1.0	83	17	1.3	4.8	Weight 2,300 gm.
" 9.....	—	1:200	0.01	6.2	5.5	0.7	88	12	1.4	7.3	
" 14.....	—	1:200	>0.02	6.6	6.4	0.2	97	3	1.5	32	Weight 2,450 gm.
" 17.....	0.1 loop intravenously.	—	—	—	—	—	—	—	—	—	
" 21.....	—	1:200	<0.003	6.1	4.6	1.5	75	25	1.6	3.0	
" 28.....	—	1:300	<0.003	6.0	4.5	1.5	75	25	1.5	3.0	Weight 2,650 gm.
" 29.....	1 loop intravenously.	—	—	—	—	—	—	—	—	—	
Mar. 2.....	—	—	—	6.6	5.0	1.6	75	25	1.5	3.0	
" 5.....	—	1:300	>0.003	6.7	4.6	1.7	73	27	1.3	2.7	
" 9.....	—	—	—	5.8	3.4	2.4	58	42	1.3	1.4	Weight 2,600 gm.
" 11.....	2.5 loops intravenously.	1:200	0.001	6.0	4.1	1.9	68	32	1.4	2.1	
" 17.....	8 loops intravenously.	1:400	0.003	6.3	5.2	1.1	82	18	1.5	4.5	48 hour culture suspended in 2 cc. of saline solution.
" 22.....	—	1:400	0.003	5.4	3.6	1.8	66	34	1.5	1.9	Weight 2,650 gm.
" 23.....	—	—	—	—	—	—	—	—	—	—	Autopsy. Marked anemia.



TEXT-FIG. 3. Immunization with living dysentery bacilli.

Experiments 4 and 5. Immunization with Living and Killed Staphylococci.—Two healthy animals were inoculated intravenously with *Staphylococcus pyogenes aureus*. One rabbit received 0.001 of a slant (2,000,000 organisms) at the beginning and two subsequent inoculations of 0.02 and 0.1 of a slant. This animal at autopsy showed an osteomyelitis of the sternum, an adhesive pericarditis, and thrombophlebitis of the deep femoral vein. The second animal was injected with cultures of staphylococci killed by heating at 60° C., for one or more hours. In all, four inoculations of 0.01, 0.2, 0.5 and 1 slant were given. The animal died on the 40th day of a septicemia following a hypopyon due to accidental injury.

Some interesting differences have been observed between the effects produced by the intravenous inoculation of living and of killed staphylococci. The injection of living organisms is followed immediately by marked fluctuations in the protein quotient (Text-fig. 3), whereas following the injection of killed organisms the globulins first diminish and then the quotient shows a gradual downward course.

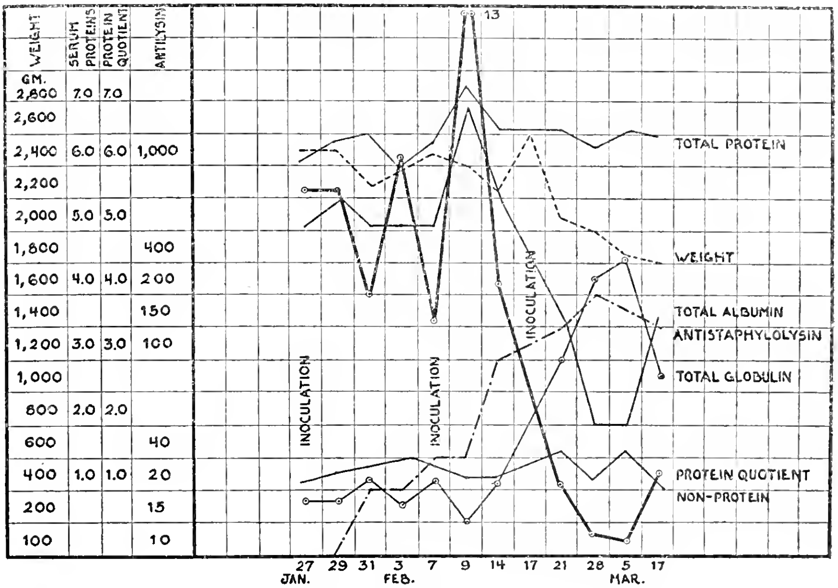
In Experiment 4 the injection of living organisms was followed on the 13th day by a marked rise in the albumin fraction. A similar

TABLE IV.

Experiment 5. Immunization with Killed Staphylococci. Rabbit 7.

Date.	Weight.	Organisms inoculated.	Antibodies.		Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
			Agglutinins.	Complement-fixing antibodies.								
	gm.				*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Jan. 27.....	2,400	0.01 slant intravenously.	0	0	5.8	3.7	2.1	64	36	1.2	1.7	
" 29.....	2,400	—	0	0	5.7	4.2	1.5	73	27	1.5	2.7	
Feb. 3.....	2,350	—	0	0	5.4	4.1	1.3	76	24	1.5	3.1	Injury on left eye.
" 7.....	2,450	0.2 slant intravenously.	1:10	<0.2	—	—	—	—	—	—	—	
" 9.....	2,250	—	1:40	<0.2	6.6	5.7	0.9	86	14	1.4	6.1	
" 14.....	2,300	—	1:100	0.2	6.6	5.9	0.7	90	10	1.4	9.0	
" 21.....	2,600	—	1:60	>0.2	5.9	4.5	1.4	76	24	1.5	3.1	
" 22.....	2,850	0.5 slant intravenously.	—	—	—	—	—	—	—	—	—	
" 28.....	2,550	—	1:100	0.05	6.7	4.2	2.5	62	38	1.6	1.6	
" 29.....	2,600	1 slant intravenously.	—	—	—	—	—	—	—	—	—	Suspension heated for 1 hr. at 60°C.
Mar. 2.....	2,600	—	—	—	—	—	—	—	—	—	—	
" 6.....	2,300	—	1:200	0.01	7.8	2.4	5.4	31	69	1.6	0.45	

event occurred also in Rabbit 7 inoculated with killed organisms. But this animal had developed a suppurative condition of the left eye about the time that this sudden rise occurred. These two observations taken in conjunction with the one following the inoculation of living dysentery bacilli suggest the possibility that such extreme fluctuations may be explained by the active multiplication of living organisms within the animal.



TEXT-FIG. 4. Immunization with living staphylococci.

Both experiments illustrate still another point which will be considered more fully later; namely, that the increase in globulins is associated with a diminution in the albumin fraction. The total proteins exhibit a slight upward course in each animal, whereas the non-protein constituents show no significant variation.

A gradual rise in antistaphylolysins took place in Rabbit 8 inoculated with living organisms. For the most part, this rise appears to parallel the increase in globulins, but we feel that there is another consideration to be kept in mind in the interpretation of this result. The experimental evidence would seem to indicate that marked

alterations in weight, such as occurred in this animal, may be associated with a great increase in the globulins of the blood independently of a rise in immune bodies.

A Comparison of the Effect of the Inoculation of Living Typhoid Bacilli upon the Normal and Immune Animal.

The observations upon the fluctuations in the serum globulins and in the antibody response in normal animals immunized with living organisms suggested the problem of the possible effect of inoculating living organisms into the typhoid immune animal. That the immunized organism because of the sensitization of its fixed tissue cells may possess a more responsive defensive mechanism is now well known (12). This power of defense may become manifest by a rapid mobilization of antibodies and by a large increase in the number of circulating leukocytes. It was our purpose in the experiments of this series to ascertain whether this protective reaction was in any manner related to the changes in the blood proteins, and more especially whether any parallelism existed between the rise in leukocytes and the increase in the blood globulins.

Experiments 1, 2, 3, 4, and 5.—Five animals already possessing a basic immunity against the typhoid bacillus were chosen for this study. In three of the animals (Rabbits 9, 10, and 4) a record was made at intervals of hourly periods of changes in the leukocytes, antibodies, and serum proteins. In one of the experiments (Rabbit 10) the observations were extended over a period of 12 days. The results of this experiment are representative of the others in this series. These are given in detail in Table V and Text-fig. 5.

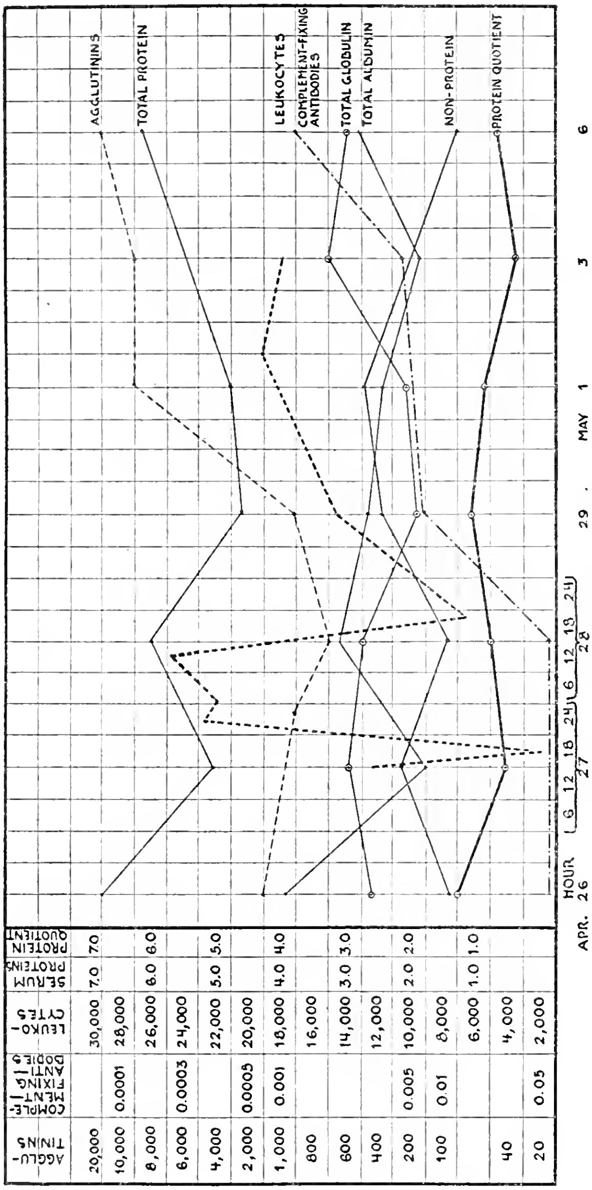
Experiment 6.—A normal rabbit weighing 2,800 gm. was inoculated intravenously with 0.25 of a slant of living typhoid bacilli (Strain H 125) on May 8. The animal tolerated the injection well, and gave a good leukocytic and antibody response. Observations on the leukocytes, antibodies, and serum proteins were made at hourly periods during the first 48 hours, and then at intervals over a period of 16 days.

The observations given in the protocols, tables, and text-figures of this series support the general conclusions that the immune animal responds more quickly to the inoculation of living organisms with a leukocytosis, a rise in immune bodies, and an increase in the blood globulins; and that the changes noted in the blood proteins bear no relation to the hyperleukocytosis.

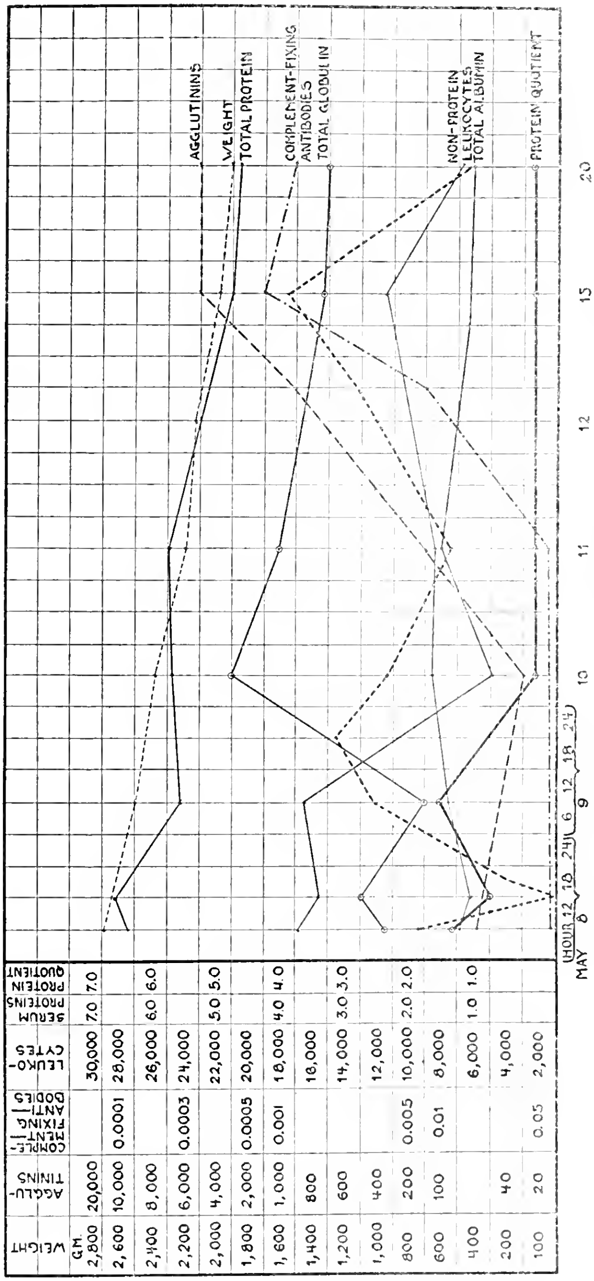
TABLE V.

Experiment 4. Rabbit 10.

Date.	Time.	Weight.	Antibodies.		Leukocyte count.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
			Agglutinins.	Complement-fixing antibodies.									
		gm.				*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Apr. 26	—	—	1: 2,000	0.05	—	7.0	4.1	2.9	60	40	1.6	1.5	
" 27	12 m.	3,475	—	—	12,300	—	—	—	—	—	—	—	Inoculation of 0.25 slant of 20 hour typhoid culture intravenously.
	4 p.m.	—	—	—	3,770	—	—	—	—	—	—	—	
	5.30 p.m.	—	1: 1,000	0.1++	—	5.3	2.0	3.3	37	63	2.4	0.6	
	8.30 p.m.	—	—	—	23,000	—	—	—	—	—	—	—	. *typhosus* in blood.
" 28	9 a.m.	—	—	—	24,600	—	—	—	—	—	—	—	" " " "
	12.10 p.m.	—	1: 800	0.05	18,600	6.2	3.2	3.0	51	49	1.7	1.0	
	6 p.m.	3,350	—	—	7,400	—	—	—	—	—	—	—	
" 29	—	—	1: 1,000	0.005++	14,900	4.9	2.8	2.1	57	43	2.6	1.3	
May 1	—	3,750	1: 10,000	0.003+++	19,700	5.0	2.7	2.3	54	46	2.7	1.1	
" 3	—	—	1: 10,000	0 003+++	19,000	5.7	2.2	3.5	38	62	2.4	0.6	
" 6	—	—	1: 20,000	0.001	—	6.3	3.0	3.3	47	53	1.5	0.88	



TEXT-FIG. 5. Injection of living typhoid bacilli. Immune animal.



TEXT-FIG. 6. Injection of living typhoid bacilli. Normal animal.

In the main the details of some of the experiments are given in Table V and Text-figs. 5 and 6, but a few points relating to the individual experiments deserve special mention. From a comparison of the results obtained in the experiments with Rabbits 11 and 12 it would appear that the rapidity of response bears some relation to the degree of the initial basic immunity. The first animal showed a fall in the agglutination titer immediately after the injection, and in this animal the rise in globulins occurred only after 72 hours. In Rabbit 12, however, the antibodies rose steadily, and in this instance a rise in the concentration of the serum globulins took place more rapidly within a period of 24 hours.

Experiments 3 and 4 are more complete since in them the observations were extended over a longer period. That the degree of hyperleukocytosis is dependent in part at least upon the number of organisms inoculated is clear from a comparison of the results obtained in Rabbits 9 and 10. In the former where the more marked reaction occurred, one-half of a standard agar slant had been inoculated; whereas, the latter received only one-quarter of a slant. The highest leukocytic reactions were observed in Rabbit 9 (44,000) and in Rabbit 4 (58,000). In this respect our observations coincide with those of McWilliams (12). The usual leukopenia which immediately follows the intravenous injection occurred in both the normal and immune animal as is graphically shown in Text-figs. 5 and 6.

By referring to these charts it will be noted that in neither animal was there any parallelism between the leukocytic response, the rise in the immune properties of the serum, and the increase in globulins. Whereas the curve showed periods in which a rise in globulins occurred simultaneously with a leukocytosis, the latter remained low even during the periods of leukopenia. Nor was any direct correspondence demonstrable between the rise in immune bodies and the increase in the concentration of the serum globulins. Both in the normal and the immune animal the latter took place long before any appreciable rise in the agglutinins and complement-fixing antibodies had occurred. In Rabbit 10, for instance, a fall in the protein quotient (globulin rise) was demonstrable within 24 hours after the inoculation at a time when the antibody content was at its lowest level; and similarly in Rabbit 13, the normal control, the serum globulins rose mark-

edly within 48 hours, while the antibodies rose to their maximum height only after 4 days. In both instances the globulin content remained high throughout the period of immunization while the antibody curve continued to rise independently of the globulins.

The advantage of such frequent determinations made at different periods during the process of immunization as compared with isolated observations made at random intervals is well illustrated in these experiments. If, for instance, a determination of the globulin fraction should show an increase at a time when the immune bodies had reached a high level, the conclusion would naturally follow that fluctuations in the two parallel one another; whereas, as we have pointed out, more frequent observations demonstrated that such parallelism was not of constant occurrence.

The tabulated results emphasize still another point of importance. To be of absolute value the albumin-globulin ratio must be expressed in terms of their quotient. This takes into consideration also fluctuations which have been found to take place in the total proteins during the course of an infection and during the process of immunization.

The Effect of the Injection of Bacterial Toxin upon the Serum Proteins.

The experimental data already presented afford evidence that both living and killed cultures of various bacteria when inoculated into an animal give rise to marked changes in the serum proteins, and more especially to an upset of the normal albumin-globulin ratio. This phenomenon may well be attributed to the changed condition of the animal's metabolism resulting from the multiplication of bacteria within its body, to the liberation of toxic products from the disintegration of the bacterial bodies, or to both causes. The part which the autolyzed bacterial bodies themselves may play in bringing about the results observed is well shown in the following experiment.

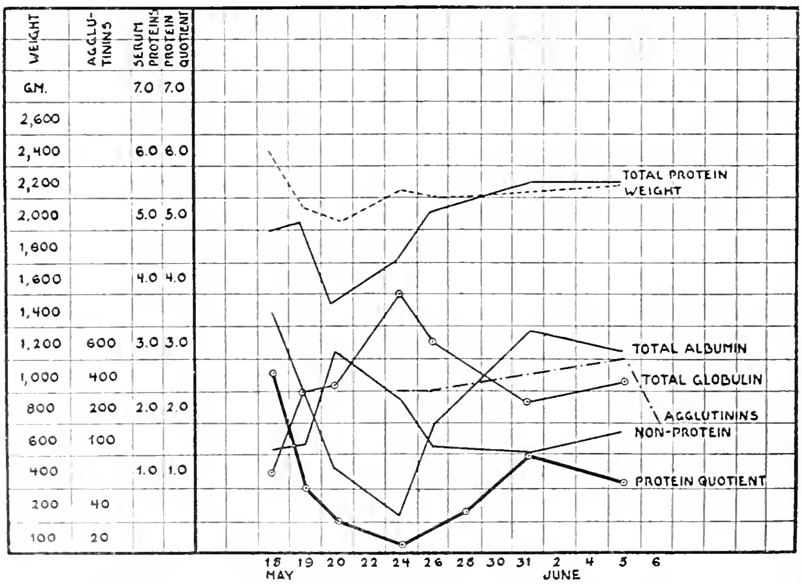
The Action of Bacterial Endotoxin.—A rabbit weighing 2,500 gm. was injected intravenously on May 18 with 1 cc. of the toxin of fowl typhoid. This toxin was prepared by growing the organisms on Martin's broth for 14 days, after which the culture was centrifugalized and filtered through a Berkefeld filter.

Specimens of 5 cc. of blood were taken 24 hours after the injection and at varying intervals until June 6. The alterations in the serum proteins following the injection are given in Table VI and Text-fig. 7.

TABLE VI.

The Effect of the Injection of Bacterial Toxin (Fowl Typhoid). Rabbit 14.

Date.	Weight.	Amount of toxin injected.	Agglutinins.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	gm.			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
May 18...	2,500	1 cc. intravenously.	—	5.0	3.7	1.3	74	26	1.6	2.8	Strain 605 grown on Martin's broth for 14 days. Centrifugalized and filtered once through a Berkefeld filter.
" 19...	2,175	—	—	5.1	2.6	2.5	51	49	1.7	1.0	
" 20...	2,100	—	—	3.9	1.3	2.6	33	67	3.1	0.5	
" 24...	2,275	—	1:400	4.6	0.6	4.0	13	87	2.4	0.15	
" 26...	2,225	—	1:400	5.3	2.0	3.3	37	63	1.7	0.6	
" 31...	—	—	—	5.7	3.4	2.3	60	40	1.6	1.5	
June 5...	2,300	—	1:600	5.7	3.1	2.6	54	46	1.9	1.1	
" 6...	—	—	1:200	—	—	—	—	—	—	—	



TEXT-FIG. 7. The effect of the injection of bacterial toxin.

The dosage of endotoxin in this experiment was apparently well chosen, for although the animal lost moderately in weight, the amount of toxin was not sufficient to prevent a gradual return of the serum proteins to a more or less normal state. The most striking effect of the inoculation, shown graphically in Text-fig. 7, was the gradual increase in the serum globulins at the expense of the albumin fraction, and a reduction in the percentage of the total proteins. This rise in globulins was already appreciable 72 hours following the injection, and 6 days later the albumin-globulin ratio still showed an inversion of the normal formula. On the 8th day the total per cent of proteins had returned to normal, and continued to rise somewhat above the normal level during the subsequent 11 days. At this time the protein quotient, however, still remained low, although the albumin-globulin ratio was beginning to revert to its normal state.

The tendency in this instance for an alteration in the blood protein fractions to readjustment is of interest. In this respect this observation is unique, for in the majority of the experiments an upset of the normal ratio continued with some fluctuations for a long period of time due to subsequent reinoculations. The readjustment of conditions in this animal may be attributed to the absence of bacterial invasion to perpetuate the process. It may be assumed that after its initial effect upon the animal, the toxin was spent, as may be inferred from the appearance of antibodies in the blood, and that the organism was then able to readjust itself.

A Comparison of the Effect of the Intraperitoneal Injection of Bacterial and of Inflammatory Irritants.

In some experiments which will be recorded at a later date, we observed that the intraperitoneal injection of red blood corpuscles gives rise to an alteration in the albumin-globulin ratio which is both rapid in its occurrence and marked in its degree. In fact such injections usually resulted in a complete inversion of the ratio within a period of 24 hours. Because we were dealing here with non-bacterial protein and with a different route of injection, it seemed worth while to ascertain whether it was the nature of the inoculated material or the route of the inoculation which was responsible for

the changes observed. With this purpose in view, the following two experiments were carried out.

Experiment 1.—On May 22 a rabbit weighing 2,525 gm. was injected intraperitoneally with 0.2 of a slant of a killed culture of staphylococci. The organisms were killed by heating for an hour at 60° C., and 1 cc. of the suspension was used. An analysis of the serum proteins was made at stated intervals following the injection. These are recorded in Table VII.

TABLE VII.

The Effect of the Injection of Killed Staphylococci Intraperitoneally. Rabbit 15.

Date.	Weight.	Organisms inoculated.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	gm.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
May 19..	2,500	—	5.8	3.2	2.6	60	40	1.7	1.5	
" 22..	2,525	0.2 slant intraperitoneally.	—	—	—	—	—	—	—	24 hour culture killed by heating for 1 hr. at 60°C. suspended in 1 cc. of saline solution.
" 23..	2,500	—	4.7	1.3	3.4	28	72	2.9	0.4	
" 24..	2,600	—	4.7	1.8	2.9	38	62	2.8	0.6	
" 26..	2,600	—	4.9	0.8	4.1	16	84	1.8	0.2	
" 31..	—	—	5.7	2.0	3.7	35	65	1.6	0.5	
June 6..	—	—	5.8	1.8	4.0	31	69	1.3	0.45	

Experiment 2.—2 cc. of an aleuronat suspension in saline solution were injected intraperitoneally into a rabbit weighing 2,150 gm. The suspension of aleuronat was so made that it corresponded in density to that of the killed staphylococci used in the first experiment. Following the injection, observations were made upon the serum proteins. These are recorded in Table VIII. The animal lost gradually in weight, and died 7 days after the injection.

Autopsy.—Small masses of unabsorbed aleuronat were found adherent to the peritoneum. There was a definite intestinal paralysis with coprostasis, chiefly in the large bowel. There was considerable injection of the peritoneum and an enteritis of the small bowel.

TABLE VIII.

The Effect of the Injection of Aleuronat Intraperitoneally. Rabbit 16.

Date.	Weight.	Amount of aleuronat injected.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	gm.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
May 19..	2,075	—	5.1	3.1	2.0	60	40	1.6	1.5	
" 22..	2,150	2cc.	—	—	—	—	—	—	—	Aleuronat (Merck) suspension made in saline solution and of about same density as that of staphylococci (Table VII).
" 23..	2,025	—	4.7	1.8	2.9	38	62	2.8	0.6	
" 24..	2,025	—	4.2	1.7	2.5	40	60	3.1	0.66	
" 26..	1,900	—	4.7	1.5	3.2	32	68	2.6	0.5	
" 29..	1,450	—	—	—	—	—	—	—	—	Death.

Both experiments would seem to support the view that the route of injection rather than the nature of the substance injected is responsible for the rapid inversion in the albumin-globulin ratio. 24 hours following the intraperitoneal injection of killed staphylococci the quotient fell from 1.5 to 0.4, indicating an increase in globulins to more than three times the initial value. This upset in the ratio continued with slight fluctuations for a period of about 2 weeks. The animal injected with aleuronat showed a change similar in every respect.

The retardation in response noted after intravenous inoculations must therefore be attributed to the protective properties of the blood which enable it to delay the action of the bacteria or toxin upon the body tissues.

Another point brought out by this experiment deserves emphasis; namely, that agents other than bacteria or their toxins may cause an upset in the serum proteins. The manner in which an inflammatory irritant and leukotactic substance like aleuronat may produce this result offers some difficulty of explanation. It is not unlikely, however, that the rapid absorption of toxic protein products result-

ing from the disintegration of leukocytes and fixed tissue cells produces a profound metabolic disturbance of which the heaping up of blood globulins is one of the resultant phenomena. A further consideration of the factors which may give rise to this result will be presented later.

DISCUSSION.

The experimental evidence presented does not support the views held by a number of workers concerning the relationship of the blood globulins to the resistance developed in bacterial infection and immunity. From a large number of observations, continued over a long period of time, we have become convinced that other causes are responsible for the rise in globulins observed in these conditions.

Our observations have shown with considerable certainty that a heaping up of globulins in the blood during the development of an infection is more apt to occur in those instances where the infection has been overwhelming and associated with extensive suppuration and wasting. We have found, in fact, that animals which succumb to such an acute process have usually developed only a moderate resistance as far as the development of immune bodies is concerned. On the other hand, a mild chronic infection may continue over a long period of time, and may register only slight changes in the blood globulins until the animal begins to emaciate and to lose in weight. This point has been discussed in connection with the tubercular and the mycotic infections.

The main points of interest have come from a study of the serum globulins during the process of immunization. Contrary to the results of a number of workers (13), our experiments have shown that immunization with bacteria causes a rise in globulins only when the animals react severely to the inoculation. Immunization carried out carefully and with a well controlled dosage is not usually accompanied by an increase in the serum globulins, although the immune bodies may attain a high concentration. The inoculation of massive doses, however, either into a normal animal or into an animal already possessing a basic immunity results in most instances in a marked rise in the globulins. This may occur, indeed, before the animal has responded by the production of antibodies. It would seem,

therefore, that no direct parallelism exists between the two phenomena. In fact we have come to regard the heaping up of serum globulins supervening during the process of immunization as an index of a metabolic disorder unfavorable to the attainment of the best immunologic results. And it is not unlikely that observations on the blood globulins may serve as an important practical guide to careful immunization.

Any attempt to explain the cause of the rise in globulins observed in infection and immunity is difficult. At best all such explanations must be of a hypothetical character until we have learned more concerning the origin of the various protein fractions, their function,[5] and their chemical nature. One important conclusion may be derived from the experimental evidence presented; namely, that the increase in blood globulins is usually accompanied by a marked metabolic disorder. This observation has been made also by other workers (14). Clinically, the metabolic disturbance is manifested by a febrile reaction, intoxication, and rapid emaciation. The extensive destruction of body protein which is going on is further evidenced by an increase in the nitrogen elimination (15). That such marked proteolytic activity may be initiated by the intravenous injection of bacteria, bacterial toxins, and protein split-products has been clearly shown by Jobling and his coworkers (16). They attribute this active proteolysis to a more or less marked mobilization of ferments, both protease and lipase. Apart from the consideration of the cause of this disturbance in metabolism, it seems reasonable to assume that it must register a change in the proteins of the blood.

More difficult to explain, however, are the facts that an inversion of the albumin-globulin ratio can be so readily produced, and that the change markedly affects the globulins. The possible explanations which may be offered for these phenomena have some basis in experiment. Moll (17) has shown, for instance, that under optimum conditions of reaction and temperature crystalline albumin can be converted *in vitro* into a substance whose chemical and physical

[5] Friedemann (*Z. Hyg. u. Infectionskrankh.*, 1910, lxvii, 279) thinks that the globulins and albumins of normal serum are in antagonism, the albumins preventing certain reactions, such as complement fixation, in which the former become active as soon as the albumins are removed or diminished.

properties correspond in every particular to a globulin. On the basis of this observation one would have to assume that the more rapid conversion of albumin into globulin within the body is only a part of the accelerated metabolism which takes place in infected and immunized animals.

This is only one of many hypotheses which might be advanced to explain the difficult questions which the recorded observations offer for consideration. But at the present time it is better to adhere to those views which have an experimental basis until further additions to our knowledge make them untenable.

SUMMARY.

The progress of an infection is usually associated with marked changes in the serum proteins. There may be an increase in the percentage of the total protein during some stage of the infection, and there is usually a change in the albumin-globulin ratio with an increase in the total globulins. This rise may antedate the development of any resistance by a considerable period of time.

The non-protein constituents of the blood show fluctuations with a tendency to rise as the infection progresses.

The process of immunization is in almost all instances associated with a definite increase in the globulins of the blood, and in some cases with a complete inversion of the normal albumin-globulin ratio. This may be produced both by living and dead organisms and by bacterial endotoxins. Massive doses usually result in an upset which shows no tendency to right itself during the period of observation.

A rise in the globulins has been shown to occur long before the animal develops immune bodies in any appreciable concentration; and where the globulin curve and antibody curve appear to parallel one another, it can be shown by a careful analysis of both curves that there is a definite lack of correspondence at various periods of the experiment.

Animals possessing a basic immunity show a more rapid rise in the globulin curve following inoculation.

There is no parallelism between the leukocytic reaction and the

globulin reaction. During periods of leukopenia the globulins may be as high as during the period of a leukocytosis.

Bacterial endotoxins produce as striking an increase in the serum globulins as do living and killed bacteria. This would seem to indicate that a bacterial invasion of the organism is not absolutely essential for the globulin changes, and that the toxogenic factor in infection and immunity must play a part in the production of the changes noted.

Inflammatory irritants injected intraperitoneally also result in a globulin increase. In this case the changes produced may best be explained by the toxogenic effect produced by the protein split products resulting from the inflammatory condition.

Intraperitoneal injections of killed bacteria give rise to a more rapid increase in the serum globulins. The rapidity of the response following intraperitoneal as compared with intravenous injections doubtless stands in intimate relationship to the neutralizing power possessed by the blood serum and perhaps to the more extensive surface of absorption following injection by the intraperitoneal route.

BIBLIOGRAPHY.

1. Brodie, T. G., A Preliminary Report of Some Experiments upon the Chemistry of the Diphtheria Antitoxin, *J. Path. and Bacteriol.*, 1896–97, iv, 460.
2. Seng, W., Ueber die qualitativen und quantitativen Verhältnisse der Eiweisskörper im Diphtherieheilserum, *Z. Hyg. u. Infections-krankh.*, 1899, xxxi, 513.
3. Hiss, P. H., Jr., and Atkinson, J. P., Serum-Globulin and Diphtheria Antitoxin. A Comparative Study of the Amount of Globulin in Normal and Antitoxic Sera, and the Relation of the Globulins to the Antitoxic Bodies, *J. Exp. Med.*, 1900–01, v, 47.
4. Ledingham, J. C. G., On the Relation of the Antitoxin to the Globulin-Content of the Blood Serum during Diphtheria Immunisation, *J. Hyg.*, 1907, vii, 65. Gibson, R. B., and Banzhaf, E. J., The Quantitative Changes in the Proteins in the Blood Plasma of Horses in the Course of Immunization, *J. Exp. Med.*, 1910, xii, 411.
5. Pfeiffer, R., and Proskauer, B., Beiträge zur Kenntniss der spezifisch wirksamen Körper im Blutserum von choleraimmunen Tieren, *Centr. Bakteriol.*, *1te Abt.*, 1896, xix, 191.
6. Pick, E. P., Zur Kenntniss der Immunkörper, *Beitr. chem. Phys. u. Path.*, 1902, i, 351, 393, 445.

7. Rodhain, J., Beitrag zur Kenntniss der wirksamen Substanzen des Antistreptokokkenserums, *Beitr. chem. Phys. u. Path.*, 1902-03, iii, 451.
8. Moll, L., Über Blutveränderungen nach Eiweissinjektionen, *Beitr. chem. Phys. u. Path.*, 1904, iv, 578.
9. Kolmer, J. A., and Trist, M. E., Studies in Non-Specific Complement Fixation. I. Non-Specific Complement Fixation by Normal Rabbit Serum, *J. Infect. Dis.*, 1916, xviii, 20.
10. Robertson, T. B., A Micro-Refractometric Method of Determining the Percentages of Globulin and Albumin in Very Small Quantities of Blood Serum, *J. Biol. Chem.*, 1915, xxii, 233.
11. Robertson, Studies in the Blood Relationship of Animals as Displayed in the Composition of the Serum Proteins. I. A Comparison of the Sera of the Horse, Rabbit, Rat, and Ox with Respect to Their Content of Various Proteins in the Normal and in the Fasting Condition, *J. Biol. Chem.*, 1912–13, xiii, 325. Wells, C. E., The Influence of Age and Diet on the Relative Proportions of Serum Proteins in Rabbits, *ibid.*, 1913, xv, 37.
12. Cole, R. I., Experimenteller Beitrag zur Typhusimmunität, *Z. Hyg. u. Infectionskrankh.*, 1904, xlvi, 371. McWilliams, H. I., Is the Hyperleucocytosis Following the Injection of Typhoid Bacilli into Immunized Rabbits Specific, *J. Immunol.*, 1916, i, 159.
13. Langstein, L., and Mayer, M., Über das Verhalten der Eiweisskörper des Blutplasmas bei experimentellen Infektionen, *Beitr. chem. Phys. u. Path.*, 1904, v, 69.
14. Cervello, C., Einfluss der Antipyretica auf die Albuminoide des Blutserums, *Arch. exp. Path. u. Pharm.*, 1910, lxii, 357. Breinl, F., Beitrag zur Kenntnis der Serumeiweisskörper, *ibid.*, 1911, lxv, 309. Glaessner, K., Ueber das Verhalten des Blutglobulins beim Immunissirungsvorgange, *Z. exp. Path. u. Therap.*, 1906, ii, 154.
15. Friedemann, U., and Isaak, S., Ueber Eiweissimmunität und Eiweissstoffwechsel, *Z. exp. Path. u. Therap.*, 1905, i, 513.
16. Jobling, J. W., and Petersen, W., Bacteriotherapy in Typhoid Fever, *J. Am. Med. Assn.*, 1915, lxv, 515.
17. Moll, L., Über künstliche Umwandlung von Albumin in Globulin, *Beitr. chem. Phys. u. Path.*, 1904, iv, 563; Kürzere Mitteilungen. 5. Zur künstlichen Umwandlung von Albumin in Globulin, *ibid.*, 1906, vii, 311.

THE RELATION BETWEEN THE THYROID AND PARATHYROID GLANDS.

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Plate 42.

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Although the thyroid and parathyroid glands are generally regarded as independent organs, numerous observations seem to indicate a functional cooperation between them. These views are based essentially on the microscopic changes that take place in one of the glands after entire or partial extirpation of the other. Although both glands under normal conditions present different and characteristic structures, they may, especially the thyroid gland, under various experimental conditions, undergo structural changes.

The opinions generally held regarding this question are as follows: (1) After parathyroidectomy hypertrophy of the thyroid gland develops (Edmunds,[1] Vassale and Generali,[2] Halpenny and Thompson[3]). (2) After complete removal of the thyroid gland with the enclosed parathyroidea interna, hypertrophy of the parathyroidea externa occurs, with a tendency to the formation of colloid alveoli; in other words, the structure becomes like that of the thyroid (Halpenny and Thompson,[3] Vincent and Jolly,[4] Blum,[5] and Kishi[6]). For these rea-

[1] Edmunds, W., Further Observations on the Thyroid Gland (VI.) *J. Path. and Bacteriol.*, 1907–08, xii, 101.

[2] Vassale, G., and Generali, F., Fonction parathyréoïdienne et fonction thyréoïdienne (Résumé), *Arch. ital. biol.*, 1900, xxxiii, 154.

[3] Halpenny, J., and Thompson, F. D., On the Relationship between the Thyroid and Parathyroids, *Anat. Anz.*, 1909, xxxiv, 376.

[4] Vincent, S., and Jolly, W. A., Some Observations upon the Functions of the Thyroid and Parathyroid Glands, *J. Physiol.*, 1905, xxxii, 65; Further Observations upon the Functions of the Thyroid and Parathyroid Glands, *ibid.*, 1906, xxxiv, 295.

[5] Blum, F., Die Schilddrüse als entgiftendes Organ, *Virchows Arch. path. Anat.*, 1899, clviii, 495.

[6] Kishi, K., Beiträge zur Physiologie der Schilddrüse, *Virchows Arch. path. Anat.*, 1904, clxxvi, 260.

sons most investigators regard both glands as cooperating physiologically, but not as identical.

Before reporting my experiments, I shall describe the changes that may occur in the thyroid that has not been subjected to surgical procedure. We know from earlier observations that the thyroid gland reacts macroscopically and microscopically to various influences. That it sometimes increases in size during pregnancy has long been known. Bircher[7] has produced hypertrophy experimentally in rats and dogs by giving them water from districts where goiter is prevalent among the inhabitants. Halsted[8] was among the first to call attention to the frequent appearance of hypertrophy of the thyroid gland in dogs, without being able to give a satisfactory explanation in all cases.

In a previous publication[9] I showed that an excessive meat diet will cause constant changes in the thyroid glands of rats. The changes consist chiefly of an increase in the weight of the glands (50 per cent), of a proliferation of the epithelial cells, and of a decrease of the colloid substance. Similar experiments with the same results have been previously performed by Watson[10] and others.

The Thyroid Gland after Meat Diet.

Thyroid glands were taken from five cats which for a period of from 8 to 15 months had lived in the laboratory entirely on a diet of meat and water. The same changes previously observed in rats occurred in the cats; *i.e.*, enlargement of the gland, proliferation of the cells, and disappearance of the colloid substance (Fig. 1).

The enlarged gland was weighed in only one instance. This animal, which weighed 3,100 gm., died during an operation as a result of the

[7] Bircher, E., Zur experimentellen Erzeugung der Struma, zugleich ein Beitrag zu deren Histogenese, *Deutsch. Z. Chir.*, 1910, ciii, 276.

[8] Halsted, W. S., An Experimental Study of the Thyroid Gland of Dogs, with Especial Consideration of Hypertrophy of This Gland, *Johns Hopkins Hosp. Rep.*, 1896, i.

[9] Tanberg, A., Om virkrungen ad ensidig kjödernoring, sorlig par glandula thyroidea, *Norsk Mag. Lægevidensk.*, 1910, viii, 516.

[10] Watson, D. C., The Rôle of an Excessive Meat Diet n the Induction of Gout, *Lancet*, 1905, i, 347.

anesthetic, after living on meat for 15 months. In this case the thyroid gland weighed 0.97 gm., and was thus more than three times as large as normally (0.25 to 0.30 gm.). In the other animals the enlargement can only be roughly estimated, but it became evident by a later operation that the thyroid gland was enlarged to about double the normal size.

The proliferation of the cells was pronounced in all cases, although the details varied microscopically. Instead of the regular alveoli lined with a single layer of cells, irregular and often ramified spaces were found. These are produced by proliferation of the epithelium which retains the character of a single layer only in a few places, and protrudes from the wall in solid broad masses, as bud-like proliferations, like partition walls of irregular and sometimes ramified forms. The more compact formations often give rise to small round cavities that probably represent the formation of new alveoli. In these cases the alveolar structure is thus fairly preserved, but in other cases one finds a proliferation which is so strong that the tissue is quite solid, and only an irregular space indicates the remains of earlier alveolar cavities. In the parts where the tissue is more compact the cells lie closely packed without any characteristic arrangement, but in some places there seems to be a tendency toward the formation of small alveoli. The increase of the interstitial connective tissue is negligible. The colloid substance had disappeared and the alveoli are filled with a transparent or slightly granular substance.

It will appear from this description that the hypertrophy caused by a meat diet corresponds with the hypertrophy that develops in the small pieces of the thyroid gland which are left after incomplete thyroidectomy. This is shown in Fig. 2, from a cat on which 10 months previously a bilateral thyroidectomy was performed with retention of both parathyroid glands. After the operation the animal was doing well, and there were no symptoms of tetany or cachexia. At autopsy small pieces of thyroid tissue the size of a pea were found.

On microscopic examination the epithelial layers are seen to have multiplied in many places and to protrude in the form of partition walls and irregular proliferations. No colloid substance was found in the alveoli.

The similarity of the two microscopic pictures is evident. The pro-

liferation of the cells seems to take place according to the same principle, and in both cases the colloid substance also disappears. It therefore seems justifiable to conclude that an excessive meat diet gives rise to an increased activity of the thyroid gland.

The Parathyroid Gland after Meat Diet.

In the animals which had been fed on meat for a long time, the parathyroid gland was also examined in order to find possible changes in the macro- and microscopic appearance. On the whole, microscopically the parathyroid gland is normal in appearance, especially as regards the arrangement of the cells. Small syncytial cells are rare, but these may also be absent in normal glands. Hence relatively large cell forms are generally observed. It should be emphasized that the cells of the parathyroid gland do not react in the same way as those of the thyroid gland on an excessive meat diet, and we may therefore conclude that there is a difference in the functions of the cells of these organs.

Whether an increase in the size of the parathyroid gland occurs cannot be determined by the experiments performed. In organs of such small dimensions and so irregular in appearance, it is difficult to ascertain whether the size is normal.

It is possible that the size of the parathyroid gland may vary according to the diet and way of living. In this connection it should be recalled that a considerable difference is found in the weight of the parathyroid gland in man and in oxen. Vincent[11] gives the average weight of each of the glands in man as 0.035 gm. I have removed the glands in oxen for opotherapeutic experiments and noted that they were considerably smaller than in man. The average weight was 0.021 gm. It is not improbable that differences in diet and metabolism may account for the relatively small parathyroid in oxen.

I have attempted to show that an excessive meat diet may develop hypertrophy of the thyroid gland. This does not mean that other causes for similar changes in the gland may not exist, and the results of surgical operations must be confirmed microscopically by serial sec-

[11] Vincent, S., Innere Sekretion und Drüsen ohne Ausführungsgang, ***Ergebn. Physiol.***, 1911, xi, 218.

tions in all cases. This is especially necessary where the larger part of the organs has been extirpated, and where it is of importance to identify all the small remaining pieces.

I shall not mention here the results of experimental extirpation of the parathyroid and thyroid glands from the clinical point of view. I shall merely state that complete parathyroidectomy always resulted in fatal tetany, and complete thyroidectomy in chronic cachexia. Fatal tetany can be prevented only by leaving part of the parathyroid gland, and part of the thyroid must be left in order to prevent cachexia thyreopriva. I shall report only the experiments which seem to indicate a mutual relation between the glands, as far as this can be determined microscopically. The investigations will therefore deal with the changes that occur in one of the organs after entire or partial extirpation of the other.

The Appearance of the Thyroid Gland after Parathyroidectomy.

When the experimental animals died of acute tetany in the course of the first few days after parathyroidectomy, no noteworthy changes were found in the structure of the thyroid gland. The gland had in all cases the same appearance as at the time of operation. The result is different when a protracted insufficiency occurs after the operation, especially in chronic tetany.[12]

In three of my experimental animals chronic tetany developed after extirpation of three parathyroid glands. The animals died after 8, 10, and 11 months, respectively, and there was thus ample opportunity of observing the results that insufficiency of the parathyroid gland would cause in the macro- and microscopic appearance of the thyroid gland. The same results were obtained in all three cases.

The thyroid gland was not enlarged; it was possibly a little smaller than normally. Microscopic examination shows that the alveoli which are normally filled with colloid substance are considerably smaller, and of a round, regular shape, and the cells are generally small and low. Compact bits of intervesicular tissue without cavities are also seen, which probably arise from the relatively small

[12] Tanberg, A., Ueber die chronische Tetanie nach Extirpation von Glandulae parathyreodideae, *Mitt. Grenzgeb. Med. u. Chir.*, 1914, xxvii, 575.

dimensions of the alveoli, whereby the section is apt to traverse compact parts of the gland.

According to this the alveoli in the thyroid gland in chronic tetany should be smaller than usual. This, in connection with the relatively small dimensions of the cells, seems to point to a lower activity of the gland by chronic insufficiency of the parathyroid gland; or, in other words, in chronic tetany the thyroid gland becomes slightly atrophied.

The following questions naturally arise in this connection: Is it not possible that this atrophy, which in all cases is slight, is caused by the chronic disease which has lasted during several months, and is it not possible that any other disease of long duration might give rise to the same changes? Is this atrophy specific? Is it due solely to the extirpation of the thyroid gland?

Before taking up these points, I shall mention a peculiarity in the microscopic picture. From the experiments described above it is seen that hypertrophy of the thyroid gland occurs after a protracted meat diet, and no hypertrophy exists in animals suffering from chronic tetany (Fig. 3). On the contrary, atrophy is evident, and yet these animals have for a period of 8 to 11 months lived on meat, which under normal conditions would be sufficient to develop the changes characteristic of this diet. Hence it appears that a meat diet does not cause hypertrophy of the thyroid gland when an insufficiency of the parathyroid gland exists at the same time.

We shall return to the first question. Is the specific cause of the phenomenon to be found in the extirpation of the parathyroid gland? The following observations from Cat 31 support the view that extirpation of the parathyroid is able to influence the structure of the thyroid gland.

Four parathyroid glands were removed on Sept. 22, 1910. After the operation a slight transient tetany ensued. The weight had increased during the last 5 months, and when the animal was killed on Oct. 28, 1911, there were no signs of disease. The cat had lived entirely on meat for 13 months.

On microscopic examination a fifth parathyroid was found enclosed in the thyroid. The thyroid was of normal size (weight 0.28 gm.). Microscopically no proliferation of the cells was found. The structure was, on the whole, of a normal appearance with regular alveoli of ordinary size filled with colloid substance.

In this case the extirpation of four parathyroid glands did not cause chronic tetany, because the fifth remaining gland was sufficient to prevent its development. But in spite of a protracted meat diet no hypertrophy of the thyroid gland occurred, and no other cause could be detected than the extirpation of the parathyroid gland which had been performed 13 months previously. It therefore may be concluded that even if extirpation of the parathyroid gland is not followed by clinically evident signs of insufficiency, a meat diet does not under these circumstances develop hypertrophy of the thyroid gland.

Cat 32 furnished even stronger evidence of this fact. This animal which had been fed on meat for a long time, was operated on Sept. 26, 1910. Two parathyroid glands and some small pieces of the thyroid were removed, which showed microscopically the characteristic signs of a meat diet. On Feb. 13, 1911, the entire right thyroid gland was removed. It presented the typical appearance of hypertrophy caused by a meat diet; *i.e.*, disappearance of the colloid substance, and a pronounced proliferation of the cells, which transformed the entire gland into a compact tissue. Hence extirpation of two parathyroids could not influence the appearance of the thyroid gland. The last operation was followed by a protracted tetany, which, however, was finally cured.

On Oct. 4, 1911, the middle third of the left thyroid gland was removed. Microscopic examination showed that the thyroid gland had lost its previous appearance. The hypertrophy caused by a meat diet had diminished, although the animal had still been fed exclusively on meat. Instead of the earlier compact proliferation small regular alveoli now appeared, being perhaps a little smaller than normally and lined with a single layer of small cuboidal cells. Colloid substance had again collected in the alveoli.

In this case microscopic pictures are seen of three different stages. At the beginning of the experiment the hypertrophy characteristic of a meat diet was present, and the extirpation of two parathyroid glands did not cause any change. After the third parathyroid gland had been removed together with one-half of the thyroid gland, symptoms of insufficiency occurred, which were finally cured. When the animal was operated on for the third time, nothing abnormal could be observed, and it became apparent that the hypertrophy had diminished and that the thyroid gland was almost normal in appearance.

The cause for this change must be found in the operation performed on Feb. 13, 1911, at which the right thyroid gland and the third parathyroid enclosed in it were removed. Extirpation of one of the thyroid glands cannot have caused the changes in appearance, as this, on the contrary, should produce hypertrophy of the remaining gland. The cause must undoubtedly be looked for in the last operation which resulted in a lasting insufficiency of the parathyroid gland. Although this had apparently been removed, the thyroid gland had nevertheless changed its structure. The conclusion may therefore be drawn that in a thyroid gland where pronounced hypertrophy is already present because of a meat diet, this disappears and the gland assumes its normal appearance after the extirpation of a sufficiently large number of parathyroid glands (in this case three).

From these examples it will be seen that extirpation of the parathyroid gland influences the microscopic appearance of the thyroid gland, and that it seems to be of comparatively little consequence whether hypertrophy caused by a meat diet is present or not. It seems to indicate that an insufficiency in the parathyroid gland corresponded to a certain picture of the thyroid, and as this picture is probably the result of a certain degree of functional activity, we come to the conclusion that an insufficiency in the parathyroid gland develops functional changes in the thyroid gland.

This insufficiency need not necessarily be pronounced enough to give clinically distinct symptoms. The thyroid gland, which easily reacts to influences leading to changes in its function (diet, pregnancy, and water from districts where goiter is prevalent among the inhabitants), is also highly sensitive to a reduction in the function of the parathyroid gland. The small alveoli with cuboidal cells and the absence of hypertrophy caused by meat diet seem to imply some loss of function, or, in other words, that an insufficiency of the parathyroid gland inhibits the functions of the thyroid gland. The view that the functions of the parathyroid and thyroid glands are specific does not, however, exclude the occurrence of a functional interaction between the two glandular systems. But it would be difficult to determine its nature and scope on the basis of these experiments alone.

Gley,[13] and more recently, Vincent, Jolly, and others have already suggested a functional cooperation which they believe to be of a vicarious nature. In our experiments, on the contrary, the results of chronic tetany, as well as the influence of parathyroidectomy on hypertrophy caused by a meat diet, seem to imply that insufficiency of the parathyroid gland causes a reduction of the functions of the thyroid gland.

The Appearance of the Parathyroid Gland after Thyroidectomy.

The material for examination was taken from three cats on which complete thyroidectomy was performed with maintenance of both external parathyroids. All the animals had cachexia thyreopriva, with apathy, fall of temperature, etc. They lived from several months up to 1 or 2 years after the operation.

On microscopic examination serial sections from two cases showed no trace of remains of the thyroid gland. In the third animal a small piece was found, hardly larger than a parathyroid gland, which probably had been instrumental in keeping the animal alive during the 3 years when it was under observation, but which had not been sufficient to prevent the development of definite symptoms of thyreopriva. The animal never showed any signs of tetany, in spite of the insufficiency of the thyroid gland, the reason evidently being that both external parathyroids were left.

In all three cases both external parathyroids were identified in serial sections. There was no essential change in their appearance. Microscopically no structure was found resembling the thyroid gland. No increase in the scant colloid substance nor any formation of follicles has been found. The appearance was normal in spite of the fact that the animals lived for a long time with a constant insufficiency of the thyroid gland, and the parathyroid, therefore, should have had ample opportunity to modify its structure and function, if it had this power.

On the other hand, the glands, as a rule, seemed to be larger than usual. In all probability a compensatory hypertrophy developed as a

[13] Gley, E., Recherches sur la fonction de la glande thyroïde, *Arch. physiol. norm. et path.*, 1892, iv, 311.

result of the extirpation of the internal glands. The actual measurements of the hypertrophy cannot be given.

No definite type of cells can be mentioned as characteristic of the hypertrophied gland. As a rule, however, the cells and the nuclei are relatively large and more symmetrical, while in the normal glands the cells are, as a rule, of various sizes, from syncytial-like cells with little protoplasm to the large transparent cells. The cells in the hypertrophied glands are always of the larger variety, and forms with transparent contents as well as forms with more granular protoplasm are to be seen. Oxyphil cells are not observed here or in the normal glands.

These experiments seem to indicate that the parathyroid gland does not undergo structural changes after total thyroidectomy.

Hypertrophy of the Parathyroid Gland.

The comparatively small changes occurring in the external glands after removal of both the internal glands and both thyroids have already been described. It would be natural to assume that these changes would be more pronounced if the animal had only one remaining parathyroid gland and had lived under a constant insufficiency, as is the case in chronic tetany.

It is also evident that the microscopic picture of the parathyroid gland as it appears in this disease does not in all cases agree with the one found in simple hypertrophy where the extirpation has not been extensive enough to give rise to a chronic insufficiency.

Cats suffering from chronic tetany show on microscopic examination that the remaining parathyroid gland may sometimes assume an appearance which does not present any point of resemblance to the previous descriptions of hypertrophied glands (Fig. 4).

Even under low magnification the microscopic aspect is pronounced in large transparent cells. Under normal conditions the parathyroid cells (Fig. 6) are comparatively little larger than those of the thyroid gland. This is also the case with the nuclei; often hardly any difference in this respect is observed. In this case, however, the contrast in the dimensions of the cells is striking, and is furthermore emphasized by the fact that the thyroid cells in chronic tetany are, if anything, smaller than usual.

With high magnification the following details can be seen: The cells are large, of round, angular, or irregular shape, and with distinct cellular limits. The nuclei are large, eccentric, and rich in chromatin; as a rule, they are round or oblong, but angular forms are frequently seen. The contents of the cells are somewhat varied, but the most characteristic feature is that the greater part of the contents, which surrounds the nuclei to a greater or less extent and often fills the entire cell, is colorless and transparent. Generally along the walls of the cells a small border of granular or filiform protoplasm is seen which without any distinct limit runs into the central transparent part. Sometimes the granular protoplasm is more distended, but even then a more transparent zone can be seen around the nucleus. Distinct vacuoles are nowhere to be seen. The vascularization is ample, and the perivascular tissue somewhat increased, so that the substance of the gland assumes the appearance of being divided into several otherwise not very distinct lobules. No round cellular infiltration is seen.

As already mentioned, the cells, as well as the nuclei, are considerably larger than those generally observed under normal conditions (Fig. 6). While the normal cells of the parathyroid glands measure from 6 to 12μ in diameter, the cells in this instance ranged from 12 by 15μ to 21 by 24μ, or about double the normal diameter. That the nuclei also considerably exceed the normal size is shown in Fig. 4.

Hypertrophy of the Remaining Parts of the Thyroid Gland.

It is well known that when large parts of the thyroid gland are removed, the remaining parts become hypertrophied. Fig. 2 shows distinctly the more characteristic features of this hypertrophy. This form of hypertrophy generally occurs in cases where the remaining parts of the gland have been so large that they are sufficient to prevent the development of cachexia thyreopriva. However, when the remaining pieces are too small to prevent these symptoms, the proliferation of the cells is more pronounced.

Fig. 5 shows the small remaining parts of thyroid tissue found in animals which have suffered for a long time from cachexia thyreopriva.

The gland is for the most part transformed into compact tissue, con-

sisting of closely packed, sharply defined cells of irregular, round, oval, or angular shapes, with nuclei of various sizes rich in chromatin. The protoplasm is also often heterogeneous, in that the cells with strongly granular protoplasm alternate with cells of more transparent and more homogeneous contents.

The arrangement of the cells is usually quite irregular. In several places it is evident, however, that they have retained a tendency to form alveoli, as one often finds small cavities which are more or less filled with granular or filiform contents or sometimes apparently empty. Surrounding these cavities, a long row of evenly arranged cuboidal cells may be observed. Colloid substance is only exceptionally found in the cavities. The tissue is pierced by an ample network of thin anastomosing strings of connective tissue, which in certain places, in the form of thick bundles, divide the gland into distinct lobules. This picture undoubtedly presents certain points of resemblance to the parathyroid gland. It differs in the more irregular shape and arrangement of the cells, in the richer development of connective tissue, and in the pronounced tendency to form small alveoli. Unless serial sections of the remaining parts of the thyroid are examined they may be mistaken for a parathyroid, which structurally shows a transition picture to the thyroid gland.

CONCLUSIONS.

The following conclusions may be drawn from the experiments presented in this article.

1. Excessive meat diet develops hypertrophy of the thyroid gland. A definite hypertrophy of the parathyroid gland under the same conditions has not been established. A meat diet does not develop hypertrophy of the thyroid gland when insufficiency of the parathyroid gland exists at the same time, even if no clinical symptoms are present. Where a pronounced hypertrophy caused by a meat diet has already developed, the hypertrophy disappears and the gland assumes its ordinary appearance after extirpation of a sufficiently large number of parathyroid glands.

2. After parathyroidectomy no hypertrophy of the thyroid gland takes place. In chronic tetany the thyroid gland seems, on the contrary, to atrophy in spite of a meat diet.

3. After complete extirpation of the thyroid gland, the parathyroid gland does not change its structure, even in cases where the cachexia lasts for several years. Small remaining parts of the thyroid gland may through hypertrophy develop into compact tissue and thereby seemingly present some points of resemblance to the parathyroid gland.

4. When the parathyroid gland hypertrophies, as in some forms of chronic tetany, this hypertrophy is characterized by the development of large, transparent, sharply defined cells, with large nuclei rich in chromatin.

5. The parathyroid and thyroid glands are independent organs, each having specific functions. This, however, does not exclude the occurrence of a direct or indirect interaction in the functions of the two systems.

6. There is reason to believe that an insufficiency of the parathyroid gland checks to some extent the function of the thyroid gland. No proof of the existence of a vicarious cooperation between the two glands has been established.

EXPLANATION OF PLATE 42.

FIG. 1. Hypertrophied thyroid gland in a cat after 15 months on a meat diet. $\times$ 150.

FIG. 2. The remaining parts of the hypertrophied thyroid gland in a cat in which cachexia did not develop after partial thyroidectomy. $\times$ 150.

FIG. 3. Atrophied thyroid gland in a cat with chronic tetany. The colloid substance present in the alveoli cannot be seen in the photograph. $\times$ 150.

FIG. 4. The parathyroid gland in a cat with chronic tetany. $\times$ 150.

FIG. 5. The remaining parts of the hypertrophied thyroid gland in a cat with chronic cachexia. $\times$ 150.

FIG. 6. Normal parathyroid gland in a cat. $\times$ 150.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

V. Further Studies on the Relation of Culture Pallida to Virulent Pallida and on Reinfection Phenomena.

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The writers have already reported studies[1] on immune sera developed in rabbits and sheep by repeated injection of treponemata cultivated from the third generation of syphilitic rabbit orchitis. Although developing powerful agglutinating and treponemicidal powers for the culture *pallida*, these sera had practically no influence on virulent treponemata obtained from lesions, and had no protective value when allowed to act on virulent treponemata before inoculation into rabbits. These studies might be interpreted as indicating one of two things. Either they show that the culture *pallida* are not identical with the virulent *pallida* and that we did not have in our hands a true *Treponema pallidum*, or that during cultivation the *pallidum* had been so changed that it had lost not only its virulence, but certain protective attributes which in the virulent state preserved it from reaction with the serum. The former possibility, though logic and the desire for the omission of no possible factor force its consideration, is not likely for a number of reasons. In the first place, the culture treponema which was used was obtained after passing for three generations through rabbits, a period sufficiently removed from the human lesion to preclude the likelihood of having obtained an organism accompanying the *pallidum* in the human lesion. Furthermore, this organism, as will be shown, is in every respect identical

[1] Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1915, xxi, 576; 1916, xxiii, 323, 341.

with the strains of *Treponema pallidum* isolated and placed at our disposal by Dr. Noguchi, and the sera which acted powerfully on this strain did so likewise for the Noguchi strains. Thus, this strain of culture *pallidum* is identical with the Noguchi strains, and these, although similarly deprived of virulence and having become subject to serum action, have in former experiments by Dr. Noguchi[2] been shown capable in their early generations of producing typical syphilitic lesions in the testes of rabbits. It has seemed more than likely to us, therefore, that the difference between the virulent and the culture treponemata was due to the fact that in its fully virulent condition, *Treponema pallidum* is in some way insulated from the defensive mechanism of the animal body. This may be due to protective structures analogous to the bacterial capsule, although we were unable by the Porges method of applying acid and heat, or by using sodium oleate as suggested by Lamar in the case of pneumococci, to render these treponemata serum-susceptible. It may, on the other hand, be due to the close biological adaptation of the virulent organisms to the animal body, whereby no reaction between the two takes place. In that case, the failure of susceptibility to antisera produced with non-virulent culture treponemata would imply a rather profound chemical change accompanying the adaptation to culture and the loss of parasitism. However this may be, the fact remains that with the sera so far produced by us, we have not been able to exert any influence on the virulent *pallida*, although the sera we have had have agglutinated the various cultures in dilutions up to 1:2,000 to 1:4,000. We have not yet been in the position to work with horses and to produce sera of highest possible potency.

In the present communication we wish to discuss further experiments bearing upon the problem of the differences between the virulent and culture treponemata, the interrelationship of various culture treponemata, and to report experiments aiming toward further comprehension of the problems of immunity in syphilis.

[2] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

Group Agglutination of Treponemata.

Incidental to the general plan of our studies, we desired to determine the specificity of the agglutination of culture treponemata in order to find out whether the sera might be used for species identification. The immunization of rabbits with culture treponemata is not a simple matter inasmuch as many rabbits die during the process. It seemed at times as though the cultures were toxic, but we have not yet been able to determine definitely whether the accidents of death were due to a true toxicity or to a slow anaphylactic poisoning such as we have noticed in cases of animals treated with many bacteria, a problem upon which we hope to report in another communication. Rabbits were treated with suspensions of culture *pallidum* washed with salt solution and heated to 56°C. for half an hour. In the early experiments we used cultures made in sheep serum rabbit kidney broth under oil; in later experiments we employed young growths obtained without animal tissue, upon coagulated egg medium with ascitic broth, a method devised in this laboratory by Miss Ruth Gilbert and referred to in a previous paper. In all cases, we need hardly add, care was taken to carry out the final serum reactions against cultures grown on animal protein different from that in the cultures with which the animals were immunized, in order to avoid error by protein-antiprotein reactions. The following experiment represents a type of the results obtained by group agglutination experiments (Table I).

TABLE I.

Experiment with Serum 624.

Treponema agglutinated.	Titer of agglutination.
T. pallidum A (homologous)	1 : 2,000
" " (Noguchi 1)	1 : 400
" " (" 2)	1 : 1,000
" *calligyrum*	1 : 4,000
" *refringens*	1 : 200
" *microdentium*	1 : 1,000
" *mucosum*	Agglutinated spontaneously in salt solution controls.

The cultures here employed, apart from *pallidum* A, are cultures placed at our disposal by Dr. Noguchi, and used in these experiments because we could be absolutely sure of their source and of their being true representatives of their respective species, being, in fact, the cultures from which these species were first described by their discoverer. The differences which seem to exist between the three culture *pallidum* strains do not seem to us to mean very much, since minor variations in the character of suspension of each strain used will often change the result to a moderate extent. The treponemata are long and often tangled, and differences almost as great as those noticed above may be due to factors not connected with the specificity

TABLE II.

Absorption of Agglutinins.

Preliminary Titration of a Rabbit Immunized with Treponema pallidum. Strain A.

Suspension of	1:10	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	Salt.
T. pallidum A	+	+	+	+	+	+	+	0	0
" *calligyrum*	+	+	+	+	+	+	+	0	0
" *refringens*	+	+	+	+	0	0	0	0	0
" *mucosum*	0	0	0	0	0	0	0	0	0
" *microdentium*	+	+	+	+	+	0	0	0	0
Tonsil organism	+	+	+	+	0	0	0	0	0

Agglutination in the Serum of the Same Rabbit (1:50) after Absorption with the Various Strains.

Tested against serum (1:50) absorbed with	Tested against suspension of			
	T. pallidum.	*T. calligyrum.*	*T. refringens.*	*T. microdentium.*
T. pallidum A	0	0	0	0
" *calligyrum*	0	0	0	0
" *refringens*	+	+	0	+
" *mucosum*	+	+		
" *microdentium*	+	+	+	0
Tonsil organism	+	+	+	+
Serum untreated	+	+	+	+
Salt solution	0	0	0	0

of the serum. For instance, in another experiment in which other suspensions of A and Noguchi 2 were used against the same serum, A, though showing a prezone, went only to 1:500, whereas Noguchi 2 went to 1:1,000. Moreover, experiments reported by us in a previous communication, have also brought out the similarity between the strains. Absorption experiments such as the ones reported here confirm the above (Table II). The close relation between the *pallidum* and the *calligyrum* is shown here.

From the preceding experiments the general impression is gained that the various strains of treponemata are closely related to each other. These observations correspond with cultural and other comparisons which are being carried out by Miss Gilbert with various strains of treponemata from many sources, studied and in part isolated by her. This work is not yet completed and will be reported in a subsequent paper. However, we believe that the work we have done indicates a close group relationship between the various microorganisms.

Action of the Serum of Syphilitic Animals and Man on Culture Pallida.

In studying the immunological problems of syphilis, one of the first hopes fostered is that of eventual success in utilizing the treponema cultures for diagnostic and therapeutic purposes. Our hope of influencing the disease in rabbits by passive immunization with culture antisera, had, of course, been indefinitely deferred by the failure of such sera to act upon or protect against the virulent organisms. However, it still seemed important to determine whether the sera of syphilitic animals and man would possess agglutinating power for the cultures. The ease and speed with which the cultures grow on the egg media might, then, supply a simple method of diagnosis, possibly analogous to the Widal reaction in typhoid. In fact, Kissmeyer[3] has recently made this claim and has expressed the belief that the method would prove of practical diagnostic value.

We may state incidentally that up to the present time, we have not found the sera of syphilitic human beings or animals to exert a considerable agglutinating or immobilizing effect upon virulent trepo-

[3] Kissmeyer, A., *Deutsch. med. Woch.*, 1915, xli, 306.

nemata from rabbits. On one or two occasions, slight differences in this respect between the sera of normal and of syphilitic animals have been noticed, but up to the present time these have been too insignificant to be convincing, and this matter will have to be studied further.

To return to the agglutination of culture organisms by the sera of infected animals and man, we cite the following experiments (Table III).

TABLE III.

Agglutination of Treponema pallidum in Rabbit Sera.

Culture Strains.

A. Normal Rabbit Sera.

1:10	1:25	1:50	1:100	1:1,000
+	0	0	0	Not tested.
+++	+	0	0	" "
0	0	0	0	" "
+++	0	0	0	" "
+++	0	0	0	" "

B. Syphilitic Rabbit Sera.

Time since first appearance of syphilitic lesion.	1:10	1:25	1:50	1:100	1:1,000
11 days	+++	++	0	0	Not tested.
27 "	+++	0	0	0	" "
19 "	+++	+	0	0	" "
2 wks.	++	++	+	0	" "
11 mos.	+++	+	0	0	" "
11 "	++	++	+	0	" "

C. Serum of Rabbit Immunized with Culture Pallida.

1:10	1:25	1:50	1:100	1:1,000
+++	+++	+++	+++	+++

It appears from this experiment and others, similar in result, that the agglutination of the culture *pallida* by the sera of syphilitic rabbits is more regular and slightly higher than that with the sera of

normal rabbits. However, circulating antibody formation is, if it exists at all, very slight, and certainly on the basis of the experiments that we have done so far, we would not venture to distinguish a syphilitic rabbit from the normal rabbit, except in the form of a conjecture, on the basis of the agglutination of the culture organisms.

The absence of antibodies in demonstrable amounts from the sera of syphilitic rabbits may be due to the fact that, in adult rabbits, generalization of syphilis is irregular and incomplete. However, we have so far been unsuccessful in producing agglutinins for the culture *pallida* by the intravenous injection of killed virulent treponemata. The virulent treponemata from lesions used in immunization were obtained by methods which we have previously described, and were killed by heating to 56°C. Two rabbits which received six injections each over a period of 7 weeks, showed no increase of agglutinins for the culture *pallida* as compared with a normal control. A third rabbit was more thoroughly treated. It received sixteen injections over a period of 10 weeks. Its serum compared with two normal controls showed apparently a slight increase in agglutinins (Table IV).

TABLE IV.

Agglutination of Culture Treponemata in the Serum of a Rabbit Immunized with a Virulent Organism.

	1:2	1:10	1:20	1:50	1:100	Control (salt solution).
Immune..............	+++	+++	+++	++	++	0
Normal 1............	+++	++	++	+	+	
" 2............	+++	+++	++	+	+	

The suspension here employed was agglutinated more readily than some other suspensions. This variation is one often noted by us and is due to peculiarities of the individual suspension, the cause for which is not entirely clear to us at present. At any rate, it is seen that the difference between the agglutination in the normal sera and in that of the rabbit immunized with dead virulent organisms is a slight one only. We are continuing experiments with sera produced in this way, but have mentioned this in passing since the observa-

tions indicate that the absence of agglutinins in syphilitic rabbits is due to the characteristics of the virulent treponemata, characteristics in which they differ from the culture organisms, rather than to the absence of generalization of the disease in these animals.

A series of similar tests has also been carried out with sera from human syphilis. This was done partly in the hope that serum antibodies might be produced in a species in which the disease is typically a systemic infection; also because we were naturally most interested in the possibility of utilizing such reactions for the diagnosis of human lues. The first experiments were done macroscopically on the sera of syphilitics giving positive Wassermann reactions. As controls the sera of normal individuals and of patients suffering from diseases not syphilitic and giving negative Wassermann reactions were used (Table V).

TABLE V.

Macroscopic Agglutinations.

	No agglutination occurred in	Agglutination occurred in serum 1:5 in	Agglutination occurred in serum 1:10 in	Agglutination occurred in serum 1:20 in
Tertiary syphilis, W. R. +, 23 cases........	13 cases.	2 cases.	5 cases.	3 cases.
Non-syphilitic diseases, W. R. −, 5 cases	5 "	0 "	0 "	0 "
Normal individuals, W. R. −, 4 cases.......	2 "	1 case.	1 case.	0 "

This series seemed encouraging in as far as syphilitic sera agglutinated more regularly than did the normal, or those of the patients with non-syphilitic diseases, and in some of the syphilitic cases the titer was considerably higher than in the normal individuals that agglutinated. However, macroscopic tests we believe to be at the present time unreliable, and in the subsequent work we set up the tests in agglutination tubes, reading them first macroscopically, but confirming these readings by microscopic examination under the dark-field microscope. Such a large number of sera from individuals without syphilis agglutinated the culture treponemata in dilutions of 1:2, that in most of our subsequent experiments we did not include dilutions lower than 1:5. The following table gives the results in cases we have tested (Table VI).

TABLE VI.

Microscopic Agglutinations.

	No agglutination occurred in	Agglutination occurred in serum 1:2 in	Agglutination occurred in serum 1:10 in	Agglutination occurred in serum 1:20 in	Agglutination occurred in serum 1:50 in	Agglutination occurred in serum 1:100 in
Primary syphilis, W. R. +, 6 cases....	3 cases.	1 case.	2 cases.	0 cases.	0 cases.	0 cases.
Secondary syphilis, W. R. +, 18 cases...	8 "	4 cases.	3 "	1 case.	1 case.	1 case.
Tertiary syphilis, W. R. +, 64 cases...	27 "	7 "	23 "	3 cases.	2 cases.	2 cases.
Tertiary syphilis, W. R. −, 2 cases....	1 case.	0 "	0 "	1 case.	0 "	0 "
Congenital syphilis, W. R. +, 4 cases....	0 cases.	1 case.	3 "	0 cases.	0 "	0 "
Non-syphilitic diseases,* W. R. −, 37 cases...	22 "	4 cases.	7 "	1 case.	2 "	1 case.
Normal individuals, 40 cases..............	35 "	4 "	1 case.	0 cases.	0 "	0 cases.

* The diseases showing high agglutination were heterogeneous, including such conditions as arthritis, tuberculosis, gastro-enteritis, glaucoma, etc., and there seemed to be no relation between disease and agglutination.

An analysis of this table yields the following summary. Agglutination in a dilution of 1:10 or above was obtained in:

Primary syphilis..................	in	2 out of	6 cases,	or 33	per cent.
Secondary syphilis...............	"	6 " "	18 "	" 33 "	"
Tertiary syphilis.................	"	31 " "	66 "	" 47 "	"
Non-syphilitic diseases............	"	11 " "	37 "	" 30 "	"
Normal persons..................	"	1 " "	40 "	" 2 "	"

Before we included non-syphilitic diseases in our plan of experimentation we were hopeful of positive results, since a small percentage of normal people agglutinated the culture *pallida* in dilutions as high as 1:10, whereas a considerable percentage of syphilitics, especially of the tertiary stage, gave such agglutinations. However, sera from various other diseased conditions, for reasons not clear to us at present, also agglutinated the culture *pallida* in a percentage not far removed

from that resulting from the syphilitic serum agglutinations. It is not impossible that some of these, because of the group reactions indicated above, may have been due to the presence of foci of spirochete infection either in the throat, teeth, or other locations in these patients. However, this is a mere assumption, and the fact remains that the occurrence of such agglutinations in non-syphilitic sera detracts considerably from any diagnostic value such reactions might have. We believe that our experiments as far as they have gone in this direction, tend to indicate a slightly increased agglutination power of syphilitic serum for the culture *pallidum*. We do not think, however, that, as at present performed, agglutination of culture *pallida* can be claimed to have any diagnostic value. These results in a general way are in harmony with the specific complement fixations obtained by Noguchi,[4] Craig and Nichols,[5] and Kolmer, Williams, and Laubaugh,[6] as well as with work done on the same problem by us by somewhat different methods.[7] The experiments, as a group, seem to indicate that if circulating antibodies for the culture *pallida* are found at all in the course of syphilis, they are in amount so small that they cannot be definitely determined by available methods. As far as our own experiments have gone up to the present time, the same is true for the virulent *Treponema pallidum*.

Attempts to Protect with Culture Pallida.

Although we had unsuccessfully tried and reported experiments in which attempts were made to protect passively with the sera of rabbits immunized with culture treponemata, we thought it worth while to carry out a few experiments in which inoculation was practiced directly on rabbits actively immunized with such cultures. The following represents an experiment of this kind (Table VII).

[4] Noguchi, *J. Am. Med. Assn.*, 1912, lviii, 1163.

[5] Craig, C. F., and Nichols, H. J., *J. Exp. Med.*, 1912, xvi, 336.

[6] Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *J. Med. Research*, 1913, xxviii, 345.

[7] In a later publication we intend to report extensively on this phase of our work.

Immunization with Culture before Inoculation.

A.

Nov. 14, 1914. Five rabbits were injected with a good suspension of sheep serum kidney cultures heated to 56°C. plus 0.5 per cent phenol. No bacteria present. Good material.

Injections were made intravenously Nov. 14, 21, and 30.

Inoculated Dec. 11 with six controls, with virulent material from a lesion in Rabbit 12.

TABLE VII.

Treated rabbits.	Injections of culture treponemata.				Results.
	Nov. 14.	Nov. 21.	Nov .23.		
	cc.	*cc.*	*cc.*		
1	3	1	1	All inoculated with virulent material, Dec. 11, 1914.	Doubtful.
2	3	1	1		+
3	3	1	1		+
4	3	1	1		Chancres.
5	3	1	1		+
Controls.					
6					+
7					+
8					+
9					+
10					Died in less than a month after inoculation.
11					

B.

Two rabbits were immunized with dead ascitic culture material, highly concentrated. One of these died in the course of immunization, the other one, No. 13, received twelve injections between Dec. 18 and Feb. 19. On Feb. 23 it was inoculated with virulent material into both testes. On Mar. 6 it developed a lesion in the left testis which was removed for other experiments,[8] and on Mar. 18 it developed a large diffuse lesion in the right.

Since these experiments take a considerable amount of time, and since, in the last case at least, extensive and satisfactory immunization with the culture *pallidum* had been practiced, we feel justified in reporting these few experiments as indicating that intravenous treatment with culture *pallida* is not likely to confer any considerable degree of resistance upon rabbits.

[8] The removal of testes for the obtaining of virulent material for rabbits is always carried out under ether anesthesia.

In the hope that localized immunization might result more favorably, we treated two rabbits intratesticularly with living ascitic egg cultures from Jan. 21, 1916, to Apr. 21, 1916, a total of eight injections being given. On May 3 they were inoculated with virulent treponemata together with two controls. On May 23 one of these rabbits developed lesions on both sides. The other one developed a lesion on June 25. One of the controls only has shown a lesion to date.

Had experiments such as those reported immediately above shown more favorable results, we should feel unjustified in publishing on the basis of so small a series. However, a negative result in an experiment of this kind is far more convincing than a positive one, and, although this work is being continued, we feel at present that it is not likely that either local or general treatment with culture *pallida* will protect.

Incidentally, the last experiment, together with two other rabbits not yet reinoculated, shows that repeated local injection of living culture treponemata will not produce a lesion, and that, as far as the rabbit testis is concerned, our cultures have lost all virulence.

Local and general immunization with suspensions of dead virulent treponemata are going on, but these are slow owing to the difficulty of obtaining material for injection and because of the frequent death of rabbits during the process. We are not yet, therefore, prepared to report on this work. However, the work of Uhlenhuth and Mulzer indicated that vaccines prepared with dead virulent material conferred no protection.

Reinfection Experiments in Rabbits.

The work of Uhlenhuth and Mulzer[9] has given us a thorough knowledge of the conditions prevailing in rabbits that have been inoculated with syphilitic virus. It is well known that in man and in monkeys a resistance is acquired during the course of syphilis which seems to protect against superinfection during a period which begins with, or shortly after, the development of the chancre, lasts throughout the secondary period, and, though waning, through the tertiary. It is also generally held that complete cure reestablishes susceptibility and that true reinfection, though rare, probably because of the relative infrequency of complete cure, is, nevertheless a fact. The extensive tabulations of John[10] may be referred to for confirmation of this statement. In rabbits the conditions are not the same, inasmuch as superinfection and reinoculation have almost invariably been suc-

[9] Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte.*, 1913, xliv, 307.

[10] John, F., *Samml. klin. Vortr.*, 1909, N. F., *Inn. Med.*, No. 157–64, 559.

cessful even during the existence of syphilitic lesions. Bertarelli[11] succeeded in reinoculating the cornea of a rabbit which had been inoculated with syphilis on a previous occasion. Uhlenhuth and Mulzer, Fontana,[12] Neisser,[13] and Pürckhauer,[14] have also found that infections of the eye were possible at a period during which the opposite eye was still syphilitic, the last named authors even showing that the cornea of the same eye could be twice inoculated. Tomasczewski[15] found that the corneal infection did not protect against scrotal infection, and, *vice versa*, although scrotal infection conferred a skin immunity. Ossola[16] and Truffi[17] also found that successful skin inoculations in rabbits made reinfection of the skin difficult although the protection so conferred was not absolute. In estimating the value of the last experiments, it should not be forgotten that successful skin inoculations in rabbits is not a thing to be obtained with regularity.

Uhlenhuth and Mulzer in extensive experiments on rabbits came to the following conclusions. (1) Syphilis of the testis and of the eye in rabbits does not protect against reinfection, and this holds good in all cases whether or not the syphilitic testicular or eye lesions have healed spontaneously, have been cured by specific drugs, or are still existing. (2) The pathological lesions resulting from such reinoculations do not differ from, and are not less severe than those following the first inoculation.

In only two animals did they notice resistance to reinoculation and these were very young rabbits that had been generally syphilized by the intracardial injection of the virus. In immunization experiments, they found that repeated injections of rabbits with the serum of rabbits intravenously treated with virulent material did not protect and that no vaccine produced with luetic material had any effect on subsequent inoculation.

In general, we may subscribe to these results of Uhlenhuth and Mulzer on the basis of our own experience in a large number of experiments. However, in one important particular, our results seem to differ distinctly from the conclusions of Uhlenhuth and Mulzer, and that is in the acquisition of a powerful localized resistance by the particular tissues which have been the seat of a syphilitic lesion at some time. After obtaining the results which we tabulate below (Table VIII), we went over the protocols of Uhlenhuth and Mulzer and found that in a number of cases reported by them, their results bear out our own, although apparently in their summaries, they overlooked the significance of their data.

[11] Bertarelli, E., *Centr. Bakteriol., 1te Abt., Orig.*, 1908, xlvi, 51.

[12] Fontana, A., *Riv. ig. san. pubb.*, 1907, xviii, 646.

[13] Neisser, A., Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911, 569; also *Arb. k. Gsndhtsamte.*, 1911, xxxvii, 569.

[14] Pürckhauer, R., *Arb. k. Gsndhtsamte.*, 1911, xxxvii, 576.

[15] Tomasczewski, E., *Berl. klin. Woch.*, 1910, xlvii, 1447.

[16] Ossola, cited by Truffi below.

[17] Truffi, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1909, lii, 555; 1910, liv, 337.

TABLE VIII.

Reinoculation of Rabbits.

Rabbit No.	1st inoculation.	Lesion.		Interval.	2nd inoculation.	Results.
		First appearance.	When healed.			
14	Nov. 6, 1914. Both testes. Strain F.	Jan. 30, 1915. Nodules in both testes.	May 10.	6 mos.	Nov. 30. Both testes. Strain A.	Negative until Jan. 11, 1916. Died.
15	July 8, 1915. Both testes. Strain S.	Aug. 25, Nodules in right testis. Left negative.	Sept. 10.	6 wks.	Nov. 30. Both testes. Strain A.	Testes negative until Feb. 23, 1916. Lost. Jan. 6. Developed keratitis in left. Treponemata found in nasal secretion. Feb. 23. Keratitis gone.
16	Apr. 2, 1915. Both testes. Strain A.	May 2. Right scrotal lesion. Nodule in left testis.	Not examined until Sept. 10. Negative.	At least 6 wks., probably much longer.	Nov. 24. Both testes. Strain A.	Negative until Apr. 10, 1916. Died. No lesions except partial loss of hair on back, Jan. 24.
17	Nov. 21, 1914. Both testes. Strain T.	Dec. 22. Nodule in left testis. Jan. 6, 1915. Diffuse orchitis in right.	Sept. 10, 1915	At least 6 wks., probably much longer.	Nov. 24. Right testis. Strain A.	Negative until Jan. 18, 1916, when doubtful nodule was noted, probably scar of the old lesion. Jan. 24, negative. Thereafter negative until Apr. 10. Died.
18	July 8, 1915. Both testes. Strain S.	Aug. 28. Nodule in left.	Sept. 10. Gone.	6 wks.	Nov. 24. Both testes. Strain A.	Negative until May 15, 1916. Died.

19	Nov. 10, 1915. Both testes. Strain F.	Nov. 24. Diffuse orchitis, left. Dec. 1. Right and left. Positive to puncture.		Less than 2 wks.	Dec. 18. Both testes. Strain A.	Jan. 12, 1916. Left testicle positive on puncture; unusually large number of spirochetes. Feb. 9. Nodule on both sides. Apr. 25. " " right.
20	Dec. 11, 1914. Intravenously immunized with culture treponemata and inoculated in both testes. Strain A.	Mar. 11, 1915. Nodule in left testis.		About a year.	Mar. 14, 1916. Left testis. Strain A.	Negative until Apr. 19, then very small nodule (left) which showed no treponemata on puncture. Died June 1. Negative.
21	May 26, 1915. Both testes. Strain A.	June 30. Diffuse orchitis in right. Sept. 2. Small nodule in left.	Jan. 24, 1916.	7 wks.	Mar. 14. Both testes. Strain A.	Doubtful nodule on right, Mar. 28. Negative on puncture. Negative within few days, then remained negative (June 20).
22	Sept. 8, 1915. Both testes. Strain L.	Oct. 4. Small nodule in left. Oct. 7. Small nodule in right and left. Nov. 26. Right excised.	Jan. 6, 1916.	2 mos.	Mar. 14. Left testis. Strain A.	Negative until May 29. Died.

TABLE VIII—*Concluded.*

Rabbit No.	1st inoculation.	Lesion.		Interval.	2nd inoculation.	Results.
		First appearance.	When healed.			
23	Oct. 21, 1915. Both testes. Strain F.	Nov. 24. Diffuse orchitis in right and left. Left removed.	Chancre until Feb. 23, 1916. Healed.	3 wks.	Mar. 14. Right testis. Strain A.	Apr. 19. Small nodule on right. Negative on puncture. Negative until June 5. Died.
24	Oct. 21, 1915. Both testes. Stain F.	Nov. 24. Nodule in left. Feb. 23, 1916. Doubtful nodule in right.	Dec. 2. Left healed.	14 wks.	Mar. 14. Both testes. Strain A.	Apr. 11. Died. No lesion.
25	Oct. 4, 1915. Both testes. Strain T.	Nov. 24. Nodule in right. Jan. 28, 1916. Nodule in left. Right excised.	Feb. 9.	5 wks.	Mar. 14. Left testis. Strain A.	Mar. 28. Doubtful small nodule. May 5. Negative on puncture. Remained negative (June 20).
26	June 9, 1915. Both testes. Strain A.	Aug. 25. Nodule in left excised. Jan. 27, 1916. Nodule in right.	Feb. 9.	5 wks.	Mar. 14. Right testis. Strain T.	Apr. 11. Distension of right testis. Negative to puncture. Apr. 23. Doubtful nodule, negative to puncture. May 23. Died. Negative.
27	Nov. 18, 1915. Both testes. Strain A (+ immune serum).	Jan. 4, 1916. Nodules in right and left. Right positive on puncture.	Feb. 23. Right healed.	3 wks.	Mar. 18. Both testes. Strain T.	Apr. 3. Chancre, right and left. Negative on puncture. Apr. 26. Nodules right and left. Negative to puncture. May 1. Died. No lesion.

28	Dec. 2, 1915. Both testes. Strain S.	Dec. 28. Small nodule in left testis. Jan. 4, 1916. Diffuse orchitis (?) in right.	Feb. 9.	5 wks.	Mar. 18. Both testes. Strain T.	Apr. 6. Chancres on right and left testes. Apr. 11. Nodules on right and left. Non-motile treponemata on puncture.
29	Dec. 6, 1915. Both testes. Strain T.	Dec. 28. Nodules in right and left. Jan. 8, 1916. Right removed.	Jan. 27.	7 wks.	Mar. 18. Left testis. Strain T.	Apr. 26. Doubtful nodule, left. Negative on puncture May 2. Negative (?) May 5. Negative on puncture.
30	Aug. 4, 1915. Both testes. Strain F.	Sept. 8. Diffuse orchitis in right and left. Right excised.	Nov. 4.	19 wks.	Mar. 18. Left testis. Strain T.	May 23. Nodule (left); many motionless treponemata.

Rabbits 14 and 15 were controlled by Rabbit 31, inoculated with the same material, which developed bilateral lesions.

Rabbits 16, 17, and 18 were controlled by Rabbit 32, inoculated with the same material, which showed bilateral lesions.

Rabbits 20, 21 22, 23, 24, and 25, were controlled by Rabbit 33, which showed a positive lesion on one side.

Rabbits 26, 27, 28, 29, and 30, were controlled by Rabbit 34, which showed bilateral lesions.

During the period of reinoculation of these rabbits (Nov., 1915, to Mar., 1916), 30 rabbits were inoculated for other purposes by the same technique. These showed 97 per cent positive results, 80 per cent of the rabbits developing bilateral lesions. In contrast to this, the above group of 17 reinoculated rabbits showed in 1, or 6 per cent, typical lesions; in 2, or 12 per cent, small lesions positive to puncture, in 8, or 47 per cent, small doubtful nodules, negative to puncture and probably never syphilitic in nature; and 6, or 35 per cent, were absolutely negative.

The protocols of Uhlenhuth and Mulzer to which we have referred[18] deal with a series of eleven rabbits inoculated in the testis and reinoculated after apparent recovery from first infection. Of this series, three, inoculated for the first time on one side only, were negative after the second inoculation. Two were inoculated first in the right, and then in the left from the lesion developed in the right testis, and therefore have no bearing on our experiments. Two were inoculated in both testes, but developed lesions only on the left. On reinoculation into both testes they developed lesions only on the right. One gave no response to the first inoculation but developed lesions on both sides after the second.

Only three out of the eleven developed lesions on reinoculation on the same side as that on which the first lesion had appeared. One of these showed a diffuse orchitis after the first inoculation but only a localized sac lesion on reinoculation. Another, although reinoculated on the left side only, subsequently developed lesions on both sides, indicating that the original infection had not yet run its course. The third, which developed diffuse bilateral lesions after the first inoculation, and a diffuse lesion on the left after the second inoculation, is the only one of the series which seems to have developed a second satisfactory lesion on reinoculation at the site of the original disease.

Thus, in a general way, although not so interpreted by them, the results of Uhlenhuth and Mulzer are not out of harmony with the results obtained by us in the seventeen rabbits reported above. Of these seventeen reinoculations, twelve failed to show lesions in testes which had shown lesions before. Another one (No. 20) developed a small nodule which was negative to puncture, but we shall not include this with the twelve as entirely negative, as we wish to be particularly cautious. The occasional small nodules which developed in the testis and were negative to puncture, in two or three of these rabbits, even if they had proved to be syphilitic by successful puncture, would indicate a considerably less extensive lesion than is ordinarily found in such inoculations, and at present we rarely fail to obtain spirochetes on puncture in syphilitic rabbits. Rabbit 27 developed chancrous lesions of the skin, therefore histologically not on the site of the previous lesion of the testis, and in this case, no treponemata could be found. Rabbit 28 undoubtedly developed small lesions positive to puncture, although the treponemata found were non-motile, an occurrence which we cannot at present satisfactorily explain. But in this rabbit, the first inoculation products were doubtful. No. 30 developed a small nodule in a testis which had

[18] Uhlenhuth and Mulzer, *Arb. k. Gsndhtsamte.*, 1913, xliv, 453.

previously been the seat of a diffuse orchitis. But the lesion was small and limited in extent and the treponemata found on puncture were motionless. One rabbit in this series (No. 19), although exhibiting definite lesions on the first inoculation, developed frank lesions after the second inoculation. It will be noticed that this is the only rabbit in which reinoculation was undertaken less than 2 weeks after apparent healing of the first lesion.

If we analyze these results together with the few data gathered from the paper of Uhlenhuth and Mulzer, they seem to us to furnish evidence that there is a distinct local resistance to reinfection developed at the site of a previous lesion. In rabbits the conditions are peculiarly favorable for experiments bearing on this point. Although generalization of the *pallida* probably occurs fairly regularly in very young intracardially inoculated rabbits, and to a limited extent in intratesticularly inoculated adults, the treponemata do not easily form lesions on other sites or active pathological reactions in many of the organs of rabbits. It is known of these animals as we have stated above that the existence of a lesion in one testis does not appear to confer resistance in other parts of the body, and we cannot speak in rabbits as we can in human beings and monkeys, of resistance to superinfection coincident with the existence of the disease. Since the experiments cited above, then, seem to point out that this resistance is conferred upon the site of pathological reaction, we are inclined to deduce that resistance to syphilis is a localized process which affects the tissues in which the reaction to the treponemata has taken place, and is not a property conferred, as in resistance to some bacterial infections, by a diffusion of antigen and a consequent production of sessile and circulating antibodies in parts remote from the point of actual infection. This complements our results and those of others who have failed to find any considerable amount of circulating antibodies, and would explain the universal failure to produce immunization either by treatment with dead syphilitic virus or, passively, with the sera of syphilitic or vaccinated human beings and animals.

The difficulty which arises in interpreting these experiments lies in determining whether the testes, after reinoculation, still harbored spirochetes from the first inoculation (the reinoculation being really

a superinfection), or whether they had completely recovered. It is practically impossible to determine this, since negative puncture is insufficient to permit conclusions as to the absence of treponemata from the entire testis. However, in some of our rabbits, 2 to 3 or 6 months, or nearly a year had elapsed, and to ordinary examinations the testis seemed normal at the time of reinoculation. We believe that the reaction capacity of the tissue cells must be the important element and not the possible persistence of a limited number of treponemata which are no longer capable of inducing reaction. As far as the analysis of the immunological condition is concerned, it is, after all, of chief importance whether the treponemata persisting or newly introduced can or cannot cause pathological injury.

CONCLUSIONS.

1. Immune sera produced in rabbits by treatment with our Culture Strain A of *Treponema pallidum* agglutinated not only the homologous strain, but also the Noguchi strains, and indicate a close group relationship of other non-pathogenic treponemata. Absorption experiments confirmed this, indicating a close relationship between the *pallidum* and the *calligyrum*.
2. Culture treponemata are not agglutinated to a much greater extent by the sera of syphilitic rabbits than they are by those of normal rabbits.
3. Culture treponemata are not agglutinated to any considerable extent by the sera of rabbits immunized with virulent treponemata.
4. The sera of syphilitic patients, especially those in the tertiary stages, agglutinate culture *pallidum* to a slightly greater extent than do those of normal individuals, but the culture *pallidum* is agglutinated to an almost equal degree by the sera of many individuals with diseases other than syphilis. We do not think that we could definitely distinguish the syphilitic from the non-syphilitic serum by the agglutination of the culture *pallida*, and therefore we do not believe that the reaction has any diagnostic value at present.
5. Immunization with culture *pallidum*, either general or local, does not seem to confer upon rabbits any considerable degree of resistance to inoculation with virulent treponemata.

6. Rabbits that have once exhibited lesions in the testis are not easily reinfected at the same site if reinoculation is practiced more than a month or so after apparent healing of the lesion. We believe that the experiments above recorded strongly suggest that resistance to syphilis in rabbits is a localized cell phenomenon not dependent upon a generalized reaction on the part of tissues remote from the site directly involved in reaction with the invading treponemata. Antibodies analogous to those formed in most bacterial infections appear in the general circulation in slight amount, if at all. The finding of many motionless treponemata in a few of the small lesions following reinoculation suggests the possibility of a purely localized formation of antibodies. This was expressed by Landsteiner some years ago when he spoke of the localized formation of agglutinins when they were absent in the general circulation.

We hesitate to apply these results too generally to the conditions prevailing in human syphilis, but they contain the possibility of an explanation for the apparent skin immunity of the secondary period, and the later successive involvement of some organs and tissues when others remain normal and when external superinfection is successfully resisted.

THE RESPIRATORY MECHANISM IN PNEUMONIA.*

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Our first investigations established the facts that the blood pressure and the vasoreflex[1] mechanism are not, as a rule, seriously impaired in pneumonia and that hearts from dogs dead of this disease contract normally when supplied with normal blood.[2] In the course of these experiments it was often observed that the respiration failed before the circulation; if artificial respiration was provided, the heart would continue to beat sometimes an hour or more after spontaneous breathing had failed. We therefore resolved to compare the condition of the respiratory mechanism in healthy cats and dogs with its condition in animals infected with pneumonia.

* Earlier papers in these studies of pneumonia are as follows: Newburgh, L. H., and Minot, G. R., *Arch. Int. Med.*, 1914, xiv, 48. Porter, W. T., and Newburgh, L. H., *Boston Med. and Surg. J.*, 1914, clxx, 125. Means, J. H., Newburgh, L. H., and Porter, W. T., *Boston Med. and Surg. J.*, 1915, clxxiii, 742; 1916, clxxiv, 464. Porter, W. T., Newburgh, L. H., and Newburgh, I., *Am. J. Physiol.*, 1914, xxxv, 1. Newburgh and Porter, *J. Exp. Med.*, 1915, xxii, 123. The first study cited consisted of measurements of the blood pressure in patients with pneumonia by Dr. Newburgh and Dr. Minot in the wards of the Massachusetts General Hospital. The remaining studies are of experimental pneumonia. These investigations, as well as the present research, were made in the Laboratory of Comparative Physiology of the Harvard Medical School, and under its direction. The cost of the present research was met in part by a grant from the Proctor Gift, contributed by the Department of Medicine of the Harvard Medical School.

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1 Porter, Newburgh, and Newburgh, *Am. J. Physiol.*, 1914, xxxv, 1.

2 Newburgh, and Porter, *J. Exp. Med.*, 1915, xxii, 123.

A method devised by Haldane and his associates may be used for this purpose. When an animal breathes through a closed system of tubes, the carbon dioxide in the inspired air progressively increases. In normal animals, this increase in the carbon dioxide stimulates the respiratory center and causes a corresponding increase in the volume of the air passing in and out of the chest. Thus, in cats and dogs, when the carbon dioxide rises to 3 per cent, the volume of the air inspired is usually doubled. The condition of the respiratory mechanism may be measured by the volume of air breathed per minute as the carbon dioxide in the inspired air rises from 1 to 5 per cent.

It will be shown in this investigation that the reaction of the respiratory mechanism to carbon dioxide is greatly diminished in pneumonia.

Method.

Cats and dogs were employed, and ether was given in all procedures that might cause pain.

The organism used was the *Bacillus pneumoniæ* of Friedländer obtained from the stock culture in the Bacteriological Laboratory[3] of the Harvard Medical School through the kindness of Dr. Sisson. This culture had been used for experimental pneumonia for 18 months or more. It was isolated originally by Dr. A. E. Steele from a patient in the Massachusetts General Hospital. The organism was passed through three guinea pigs to increase the virulence to such a degree that 1 cc. of a broth culture injected into the peritoneum killed a guinea pig in 12 hours. The virulence was maintained at this point by occasional passage through additional guinea pigs. Some months after beginning the research, the stock culture was rejuvenated by a bacillus from a case of Friedländer's septicemia, for which we are indebted to Dr. Steele.

Pneumonia was produced by injecting into the trachea broth cultures incubated from 18 to 24 hours. The quantity injected varied;

[3] We are indebted to Dr. Calvin C. Page for the bacteriological examinations of the culture. He found that the culture corresponded morphologically and biologically with published descriptions of *B. pneumoniæ*. Nine different sugars were used for fermentation. The two strains fermented the same sugars, but the amount and time of maximum gas formation varied slightly.

we found that 2 cc. per kilo of body weight caused death in dogs usually in about 30 hours. The dogs were given subcutaneously from 0.5 to 1.0 per cent of a 3 per cent solution of morphine sulphate, to lessen or prevent coughing. The culture was injected after the method of Lamar and Meltzer.[4] In cats, owing to the small size of the bronchus, the injection was made into the trachea near the bifurcation.[5]

The course of the disease may be seen from the following protocols.

Dog 9.—Nov. 28, 1915. 11.30 a.m. Inoculated.

Nov. 29, 9 a. m. Rectal temperature 39°C. Breathing labored and rapid. Walks about easily. Restless.

4 p.m. Temperature 39°C.

6 p.m. Temperature 37°C. Unsteady.

7.30 p.m. Temperature 36°C. Lies on one side. In coma.

8.10 p.m. Convulsion. Breathing stops. Artificial respiration.

8.45 p.m. Apex beat of heart can still be seen. No corneal reflex.

9.30 p.m. Heart beat ceases.

Dog 14.—Dec. 10, 1915. 10 a.m. Inoculated.

Dec. 11, 9 a.m. Temperature 40°C. Respiration rapid and labored. Can stand, but prefers lying down. Restless.

10.30 a.m. Temperature 39°C.

1.30 p.m. Temperature 38.2°C. Able to stand.

3.00 p.m. Temperature 38°C. Cannot stand or sit up.

4.10 p.m. Temperature 37°C. Coma.

6.45 p.m. Temperature 35°C. Corneal reflex present.

8.20 p.m. Temperature 35°C. Corneal reflex absent.

8.30 p.m. Death.

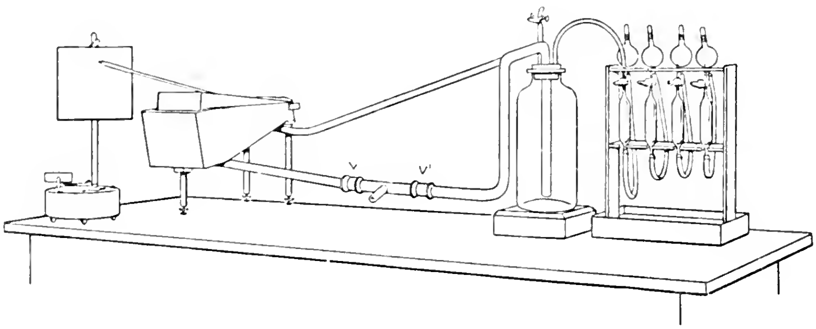
Bacillus pneumoniæ, used as we have described, is practically always fatal. The fatal issue is almost invariably preceded by a characteristic fall in temperature. This fall is so constant as to form an admirable prognostic sign, of much value when it is desired to measure the respiratory reaction shortly before death. Thus it is possible to separate the animals into two groups, those whose tem-

[4] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 135.

[5] As far as we are aware, acute pneumonia in cats was produced first by Porter and Newburgh (*Boston Med. and Surg. J.*, 1914, clxx, 125). It was produced in dogs by Kinjoun and Rosenau (*Bull. Hyg. Lab., U. S. P. H.*, 1897, 762) and by Lamar and Meltzer (*J. Exp. Med.*, 1912, xv, 133).

perature is above 37.5°C. and those below this level. The latter animals were always near death.

The measurements to be presented in this investigation were made only on cats and dogs which afterwards died of pneumonia, and in which an autopsy showed the presence of typical consolidation.

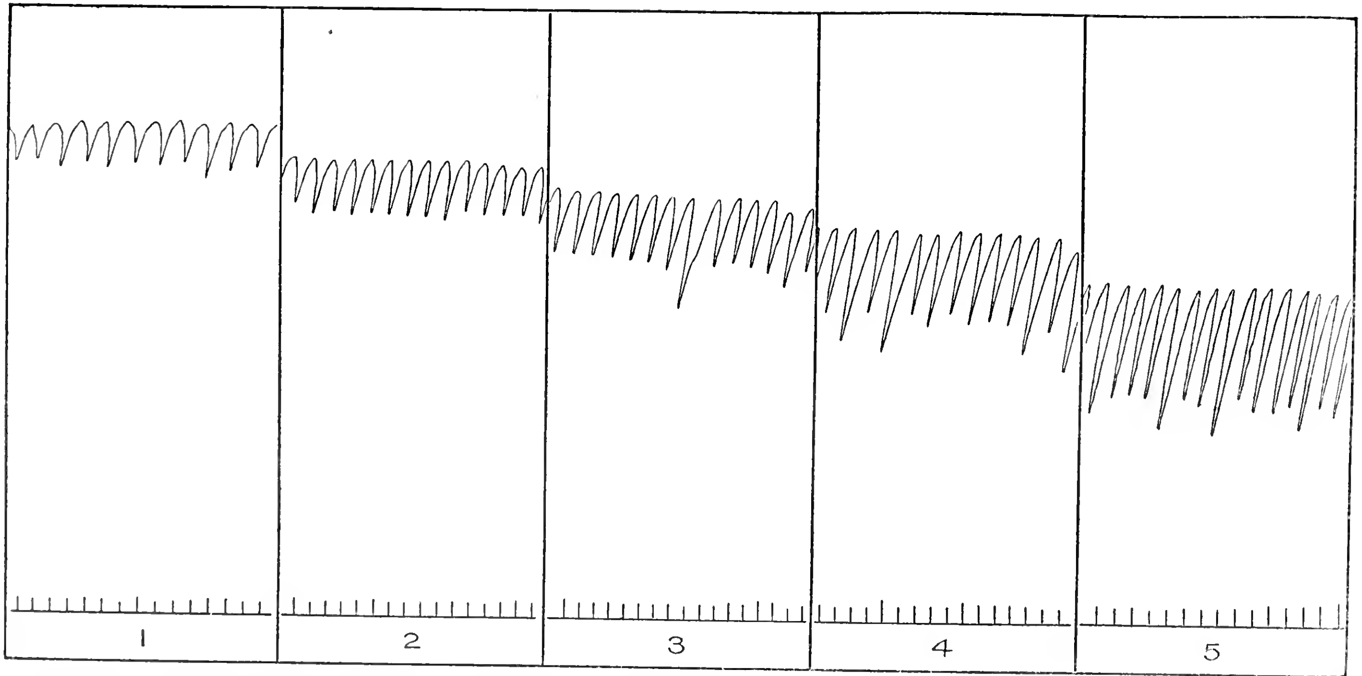


TEXT-FIG. 1. The apparatus. From left to right: the kymograph; the spirometer; the Tissot valves, v, v'; the bottle; the mercury sample tubes. The electromagnetic time signal and the Haldane apparatus are not shown.

A portion of the apparatus employed is shown in Text-fig. 1. The tracheal cannula of the animal in which the respiratory reaction was to be measured was joined to a rubber tube placed between two Tissot[6] valves connected in such a way that the animal breathed into a spirometer[7] and out of a bottle connected in its turn with the spirometer, so that the lungs, the spirometer, the bottle, and the connecting tubes formed a closed system. Evidently by this arrangement the volume of the air passing into and out of the chest was recorded by the spirometer, while the carbon dioxide exhaled by the animal constantly accumulated in the closed system. Samples of the air thus enriched with carbon dioxide were withdrawn at frequent intervals by the mercury tubes shown in Text-fig. 1. As each sample was taken, a mark was made on the spirometer record above the time record (the electric time signal is not shown in Text-fig. 1). As the carbon dioxide in the respired air increased, the spirometer curve became deeper, until the maximum reaction was reached (Text-fig. 2).

[6] Metal flap valves so lightly constructed as not to impede respiration.

[7] This quantitative aeroplethysmograph was invented by Professor J. Gad.



Text-Fig. 2. Apr. 13, 1916. Volume of tidal air in a normal dog breathing 1, 2, 3, 4, and 5 per cent of carbon dioxide. The time curve marks alternate seconds. The gradual descent of the curve is due to the gradual diminution of oxygen in the closed system. This dog was normal save for a tracheotomy tube; it was trained to stand quietly during the experiment. In Text-fig. 3, this curve is plotted with the curve of a normal decerebrate dog.

The spirometer was empirically graduated by recording the vertical rise of its writing point with each increase of 50 cc. in its contents. When the experiment was finished, the empirical graduation scale was used to measure the number of cubic centimeters breathed in the minute nearest the mark at which the air sample was withdrawn. The air in the sample was analyzed for carbon dioxide in a Haldane apparatus and the percentage written against the ventilation (Table I). Thus the cubic centimeters breathed per minute as the carbon

TABLE I.

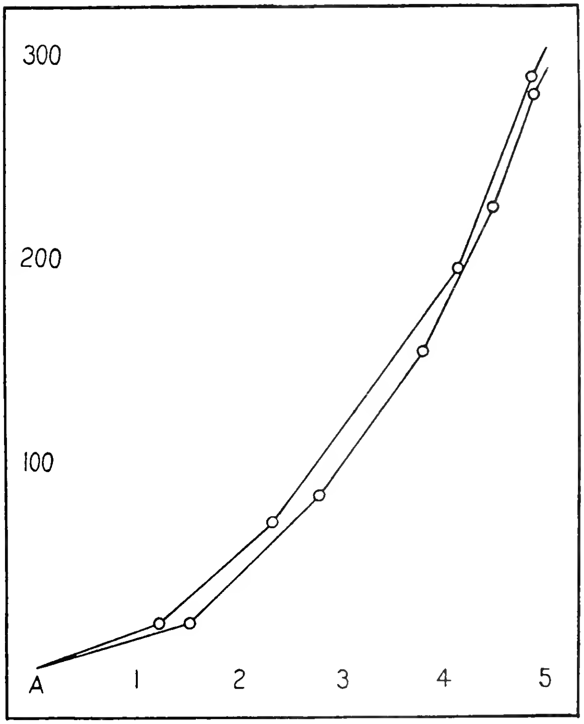
The Reaction of the Respiratory Mechanism to Carbon Dioxide in the Normal Decerebrate Dog (No. 2 A).

Nov. 19, 1915.

Carbon dioxide in the inspired air.	Respirations per min.	Volume of each respiration.	Tidal air per min.	Percentile increase in tidal air.
per cent		*cc.*	*cc.*	*per cent*
Room air.	26.5	200	5,300	
1.50	30.0	215	6,450	21
2.74	35.0	280	9,800	85
3.73	36.0	375	13,500	155
4.46	38.0	450	17,100	223
4.86	45.0	450	20,220	282
5.28	48.0	465	22,350	320

dioxide rose from the atmospheric level to 5 per cent or more were recorded accurately. When plotted on coordinate paper after the manner of Peabody,[8] as in Text-fig. 3, a curve results, expressing the reaction of the respiratory mechanism to increasing quantities of carbon dioxide. Upon this curve may be read the cubic centimeters breathed per minute at 1, 2, 3, 4, and 5 per cent of carbon dioxide. The data for Table II and Tables IV to XI were obtained in this way.

[8] Peabody, F. W., *Arch. Int. Med.*, 1915, xvi, 851.



TEXT-FIG. 3. Volume of tidal air in two normal dogs; the upper curve without decerebration; the lower with decerebration. The data for the decerebrate curve are given in Table I. The numbers at the left of the figure give the percentile increase in tidal air; the numbers below give the per cent of carbon dioxide.

TABLE II.

The Effect of Ether upon the Respiratory Reaction to Carbon Dioxide in the Dog.
Apr. 13, 1916.

Carbon dioxide in the inspired air.	Percentile increase in tidal air per min.	
	20 min. after ether.	35 min. after ether.
per cent	*per cent*	*per cent*
1	15	18
2	28	56
3	50	118
4	102	190
5	152	302

In certain dogs, ether was used for some operation preliminary to the carbon dioxide test. Ether lowers the irritability of the respiratory mechanism. Thus in the experiment of April 13, 1916, a dog weighing 4.5 kilos was etherized and tracheotomized at 10.45 a.m. At 11.05 and at 11.20 a.m. the carbon dioxide reactions were measured. They were as shown in Table II. The depression of the respiratory mechanism from ether is very evident. Where ether is used, time must be allowed for its elimination.

It need hardly be said that before each experiment the spirometer, bottle, and connecting tubes were washed free of carbon dioxide, except that in room air. Since the method consists of a comparison of the volume breathed per minute in atmospheric air with that breathed when the air contains increasing quantities of carbon dioxide, it is important that the initial or atmospheric ventilation should be accurately recorded, and the animal must therefore be kept quiet. Ether is, of course, excluded, as we have already pointed out. It is known that morphine and chloral also depress the respiratory center. Urethane cannot be used, as is shown by the experiment recorded in Table III. It appears from Table III that with urethane the

TABLE III.

The Effect of Urethane upon the Respiratory Reaction to Carbon Dioxide in the Cat. Sept. 15, 1915.

Carbon dioxide in the inspired air.	Respirations per min.	Volume of each respiration.	Tidal air per min.	Percentile increase in tidal air.
per cent		*cc.*	*cc.*	*per cent*
Room air.	52	19	987	
0.60	50	22	1,100	11
2.05	48	26	1,249	26
3.41	50	32	1,600	62
4.45	53	35	1,855	88
5.18	55	41	2,255	128
5.94	59	48	2,830	187

cubic centimeters breathed per minute when the carbon dioxide in the inspired air was 5 per cent had increased in the urethane cat only about 120 per cent, whereas, reference to Table VIII will show that the normal increase should be about 285 per cent.

Not being able to keep certain animals absolutely quiet with a drug that would not depress the respiratory center, we resorted in these cases to decerebration under ether anesthesia. The pons was divided by a seeker sufficiently blunt to prevent injury to the blood vessels. Time was allowed for elimination of the ether necessary to produce narcosis. Both the normal and the pneumonic cats were decerebrated. The normal dogs were also decerebrated, but the pneumonic dogs were measured after the disease had made them lethargic or comatose.

All the animals in which the respiratory function seemed injured by decerebration were excluded. As a rule, the respiration and circulation are wholly normal after decerebration. The animals often lived many hours after this operation. Cats lived several days. Nevertheless, it seemed desirable to make sure by a quantitative test that decerebration did not depress the respiratory mechanism. An additional reason was that no one, as far as we are aware, had previously measured the carbon dioxide reaction in a normal or in a decerebrate dog. On April 13, 1916, we accordingly measured the carbon dioxide reaction in a dog wholly normal except for a tracheotomy tube. The increases in tidal air as the inspired carbon dioxide rose from 1 to 5 per cent were 18, 56, 118, 190, and 302 per cent, respectively. These are compared in Table IV with the averages

TABLE IV.

The Respiratory Reaction to Carbon Dioxide in a Normal Dog (Tracheotomy Only) and in Normal Decerebrate Dogs.

Carbon dioxide in the inspired air.	Percentile increase of tidal air in a normal dog.	Average percentile increase of tidal air in decerebrate dogs.
per cent	*per cent*	*per cent*
1	18	19
2	56	48
3	118	96
4	190	182
5	302	285

obtained from normal decerebrate dogs. It is obvious that decerebration does not impair the reaction of the respiratory mechanism to carbon dioxide.

We pass now to a general criticism which at first seems important but which upon reflection is easily proved fallacious. Since the volume of the tidal air in animals with pneumonia is often greater than the tidal air in normal animals, it might be supposed that pneumonia animals do not increase their respiration when stimulated by carbon dioxide because they are already breathing as much as they can. That this is not a source of error is demonstrated by comparing the average carbon dioxide reaction of animals whose tidal air at the beginning of the observation has in every case approximately the same volume. When the carbon dioxide in the inspired air was 2 per cent:

4 normal cats breathed............................1,233 cc. per min.
4 pneumonia cats breathed........................1,232 " " "

Thus the tidal air in both groups was practically equal in volume.

When the carbon dioxide in the inspired air rose to 4 per cent, the same

4 normal cats breathed............................2,330 cc. per min.

but the

4 pneumonia cats breathed........................1,758 " " "

Thus at 2 per cent the pneumonia cats and the normal cats breathed alike, while at 4 per cent the pneumonia cats had increased less than half as much as the normal cats.

The detailed observations of the four normal and four pneumonic cats are set forth in Table V.

TABLE V.

Pneumonic Cats and Normal Cats May Breathe Practically the Same Volume of Air per Minute at the Beginning of the Test; but when the Inspired Carbon Dioxide Rises, the Pneumonic Ventilation Increases Much Less than the Normal; the same is true of Pnuemonic Dogs.

No. of animal.	Percentile increase in cc. breathed per min. as the inspired carbon dioxide increases from 1 to 4 per cent.			
	1 per cent.	2 per cent.	3 per cent.	4 per cent.
Pneumonic cats.	*cc.*	*cc.*	*cc.*	*cc.*
18 B	1,160	1,350	2,020	2,780
22	1,190	1,295	1,430	1,490
23 C	1,070	1,165	1,345	1,720
25*	1,070	1,080	1,090	1,040
Average.......	1,123	1,223	1,471	1,758
Normal cats.				
1 A	1,080	1,360	1,930	2,450
5 B	1,045	1,150	1,250	1,620
6 C	960	1,180	2,020	2,950
15 A	1,005	1,240	1,680	2,300
Average.......	1,023	1,233	1,720	2,330

* Cat 25 died at the close of this experiment; the observation was excluded from our final results (Table IX) because it was so favorable to our contention.

Similar results were obtained with dogs. Three dogs, with marked symptoms of pneumonia, breathed atmospheric air at the average rate of about 5,000 cc. per minute; at 5 per cent of carbon dioxide, they increased the air inspired per minute by 85 per cent. Three normal dogs also breathed about 5,000 cc. per minute; at 5 per cent of carbon dioxide the normal dogs increased the air inspired by 216 per cent. The data for these dogs, three normal and three very ill with pneumonia, are found in Table VI.

TABLE VI.

Three Normal Dogs and Three Very Ill with Pneumonia. The Initial Ventilation Is Practically the Same, but when the Carbon Dioxide in the Inspired Air Rises to 5 Per Cent, the Pneumonic Dogs Give Scarcely One-Third the Normal Reaction.

No. of animal.	Initial ventilation per min.	Percentile increase in cc. breathed per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
		1	2	3	4	5
	cc.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Normal decerebrate dogs.						
2 A	5,300	14	47	103	181	293
3 B	4,160	10	22	80	131	
16 A	5,360	10	26	63	125	185
16 B	5,320	31	77	137	204	250
16 C	5,800	20	42	69	97	135
Average......	5,188	17	43	90	148	216
Pneumonic dogs.						
9 D	4,500	15	20	25	33	37
10 B	6,375	11	42	83	94	97
11 A	5,180	19	44	64	94	122
Average......	5,352	15	35	57	74	85

Additional evidence that the failure of the respiratory mechanism in pneumonia is not due to the encroachment on the respiratory space in the lung is found in the observation that the area of consolidation may remain unchanged while the impairment of the respiratory mechanism increases progressively. The respiratory distress increases while the percussion dullness does not extend.

Moreover, if the failure to react were due to the loss of respiratory space from consolidation of the lung, large consolidations should be accompanied by a large reduction in the carbon dioxide reaction, and small consolidations by small reductions. This is not the case; there is no parallel between the area of consolidation and the amount of the reaction.

Similar evidence may be obtained by injecting starch into the lungs. We have by such injections solidified areas as large as those consolidated in bacterial pneumonia, but in these starch pneumonias

the carbon dioxide reaction was almost normal. The details of these experiments are presented in Table VII.

TABLE VII.

The Reaction to Carbon Dioxide in Dogs in Which Portions of the Lung Were Consolidated by Injecting Starch into the Bronchi.

No. of animal.	Date.	Initial ventilation per min.	Initial respirations per min.	Percentile increase in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent. 1	2	3	4	5	Area of consolidation.
	1916	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
20	Jan. 27	3,008	37	25	53	90	140	220	Right lower and middle lobes.
21	" 31	5,400	45	30	60	89	125	183	Right lower lobe does not expand under air pressure; small part of right middle lobe also consolidated.
23	Feb. 4	3,500	45	20	40	55	117	250	Fully half the lung does not expand when air is blown into trachea.
25	" 5	4,310	72	45	65	110	155	250	Right lower and half of right middle lobes do not distend with strong air blast.
Average....		4,054	49	30	54	86	134	226	

Again, evidence against a mechanical explanation of the observed depression of the respiratory mechanism is afforded by the measurements obtained by Dr. Means from a case of artificial pneumothorax. In this patient, a woman, the x-ray showed a nearly complete collapse of the left lung, from an operation 2 years before this examination. The vital capacity, which had been 2.1 liters before pneumothorax was produced, had fallen to 1.3 liters. The average of two determinations was an increase in total ventilation of 115 per cent when breathing 5 per cent carbon dioxide, and 162 per cent when breathing 6 per cent. A reference to Peabody's[8] results will show that this is normal.

Finally, we call attention to the fact that as the disease advances, the carbon dioxide reaction diminishes. If this were due to an increase in the volume of air breathed per minute at the beginning of each successive test, this initial volume should be greater as the

carbon dioxide reaction falls off, but the contrary is the case; the initial ventilation lessens with the lessening of the reaction. Dog 6 is an example of this observation.

Dog No.	Experiment.	Hrs. before death.	Initial ventilation per min.	Increase at 3 per cent carbon dioxide.
			cc	*per cent*
6	A	5	18,630	106
6	B	3	13,800	60
6	C	½	10,400	38

We conclude therefore that our method cannot be impugned on the ground of a lessening of respiratory space in the pneumonic lung.

The Reaction of the Respiratory Mechanism in Pneumonic Cats and Dogs.

TABLE VIII.

The Respiratory Reaction to Carbon Dioxide in Normal Decerebrate Cats.

Cat No.	Experi-ment.	Date.	Initial ventilation per min.	Initial respirations per min.	Percentile increase in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
					1	2	3	4	5
		1915	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	A	July 21	485	16	11	55	125		
1	B	" 21	473	17	13	38	115	245	
1	C	" 21	422	17	20	47	112	254	433
1	D	" 21	900	40	20	50	116	174	
2	A	" 22	585	18	13	30	74	160	410
2	B	" 22	289	6	5	10	35	98	206
4	A	Aug. 20	471	7	8	17	25	64	124
4	B	" 20	444	7	2	15	32	53	80
5	A	" 25	741	57	22	46	96	152	205
5	B	" 25	820	82	20	30	53	97	162
5	C	" 25	695	58	32	77	121	173	327
6	A	" 24	657	35	36	64	96	151	260
6	B	" 24	764	33	7	26	55	101	213
6	C	" 25	845	25	13	38			361
7	A	" 24	3 2	18	30	86	124	193	345
7	B	" 24	492	21	20	40	68	134	206
7	C	" 25	256	11	26	64	170	293	370
7	D	" 25	294	14	46	86	136	222	
15	A	Sept. 22	776	37	30	58	112	195	348
15	B	" 22	496	35	27	58	104	180	366
15	C	" 22	319	15	12	37	106	225	253
17		" 23	238	9	24	61	131	248	430
Average.			539	26	20	47	96	171	283

TABLE IX.

The Respiratory Reaction to Carbon Dioxide in Pneumonic Decerebrate Cats.

Cat No.	Experiment.	Date.	Initial ventilation per min.	Initial respirations per min.	Percentile change in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
					1	2	3	4	5
		1915	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
10	A	Sept. 7	1,171	78	22	40	54	134	
10	B	" 7	1,767	93	15	27	46		
11	A	" 10	4,300	216	0	6	18		
11	B	" 10	3,000	158	14	26	33	54	
18	A	" 30	1,511	54	14	39	88	134	
18	B	" 30	1,023	46	13	33	98	170	
18	C	" 30	1,025	38	11	44	81	118	112
22		Oct. 13	1,100	55	8	18	30	35	65
23	A	" 13	1,770	59	9	19	29	36	34
23	B	" 13	1,485	55	0	1	7		
23	C	" 13	1,034	36	3	13	31	67	95
26	A	" 16	2,600	65	6	12	25	43	58
26	B	" 16	1,809	67	8	17	26	27	28
26	C	" 16	1,682	40	0	0	6	8	2
Average..................			1,806	76	9	21	41	75	56

The observations on normal and pneumonic cats are given in Tables VIII and IX. They may be summarized as follows:

Carbon dioxide in the inspired air.	Increase in volume of tidal air in normal cats.	Change in volume of tidal air in pneumonic cats.
per cent	*per cent*	*per cent*
1	20	9
2	47	21
3	96	41
4	171	75
5	283	56

It is clear that the reaction of the respiratory mechanism to carbon dioxide is greatly diminished.

The greatly impaired reaction of the respiratory mechanism in pneumonia is shown also by a comparison of the normal reaction with (*a*) that of cats moderately ill with pneumonia and (*b*) that of cats near death with this disease. When the carbon dioxide in the inspired air rose to 3 per cent the volume of the tidal air increased:

	per cent.
In normal cats.	96
In cats moderately ill with pneumonia.	48
" " [9] near death " "	{ 12 24

TABLE X.

The Respiratory Reaction to Carbon Dioxide in Normal Decerebrate Dogs.

Dog No.	Experiment.	Date.	Initial ventilation per min.	Initial respirations per min.	Percentile increase in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
					1	2	3	4	5
		1915	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	A	Nov. 17	3,720	18.6	27	54	96	290	
1	B	" 17	2,828	24.0	12	42	80	120	370
2	A	" 19	5,300	26.5	14	47	103	181	293
3	A	" 22	3,490	36.0	17	34	63	174	305
3	B	" 22	4,160	54.0	10	22	80	131	
16	A	Dec. 14	5,360	25.5	10	26	63	125	185
16	B	" 14	5,320	28.0	31	77	137	204	250
16	C	" 14	5,800	23.2	20	42	69	97	135
17	A	" 15	3,880	36.9	44	96	174	314	457
17	B	" 15	3,590	25.6	4	42	100	181	
Average.			4,345	30.0	19	48	96	182	285

TABLE XI.

The Respiratory Reaction to Carbon Dioxide in Pneumonic Dogs.

Dog No.	Experiment.	Date.	Hrs. before death.	Rectal temperature.	Initial ventilation per min.	Initial respirations per min.	Percentile increase in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
							1	2	3	4	5
Temperature above 37.5° C.											
		1915		°C.	cc.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
6	A	Sept. 27	$5\frac{1}{2}$	38	18,630	162	22	63	106		
9	A	Nov. 29	8	39	5,400	30	24	87	148	178	211
9	B	" 29	4	39	5,400	30	24	83	127	144	152
10	A	" 30	6	39	4,940	77			76	108	153
14	A	Dec. 11	9	39	7,910	72	10	24		104	175
14	B	" 11	6	38.2	11,280	83.5	5	10	17	25	50
14	C	" 11	$4\frac{1}{2}$	38	10,090	84	4	8	12	13	19
Average.			6	38.6	9,093	77	15	46	81	95	127

[9] This group includes two cats that died at the close of the observation. If these are excluded, the increase becomes 24 per cent instead of 12 per cent.

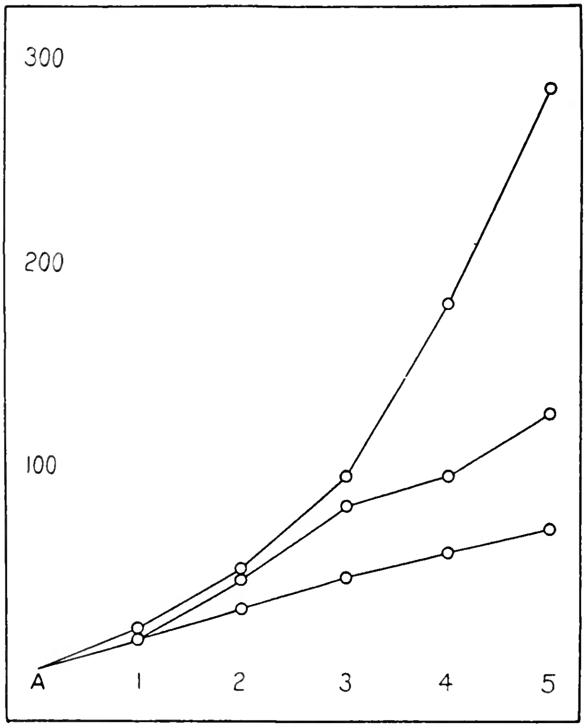
TABLE XI—*Concluded.*

Dog No.	Experiment.	Date.	Hrs. before death.	Rectal temperature.	Initial ventilation per min.	Initial respirations per min.	Percentile increase in tidal air per min. as the inspired carbon dioxide increases from 1 to 5 per cent.				
							1	2	3	4	5
Temperature 37.5° C. and below.											
		1915		°C.	cc.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
6	B	Sept. 27	3½	35	13,800	138	8	28	60		
6	C	" 27	½	33.5	10,400	130	18	34	38		
9	C	Nov. 29	2	37	6,720	160	31	57	70	72	74
9	D	" 29	¾	36	4,500	150	15	20	25	33	37
10	B	" 30	2	37	6,375	85	11	42	83	94	97
11	A	" 29	1	34	5,180	115	19	44	64	94	122
14	D	Dec. 11	3	37	10,320	76.5	2	4	12	20	25
14	E	" 11	1½	35	9,590	68.5	6	12	17	39	50
Average			1¾	35.6	8,361	115	14	30	46	59	68

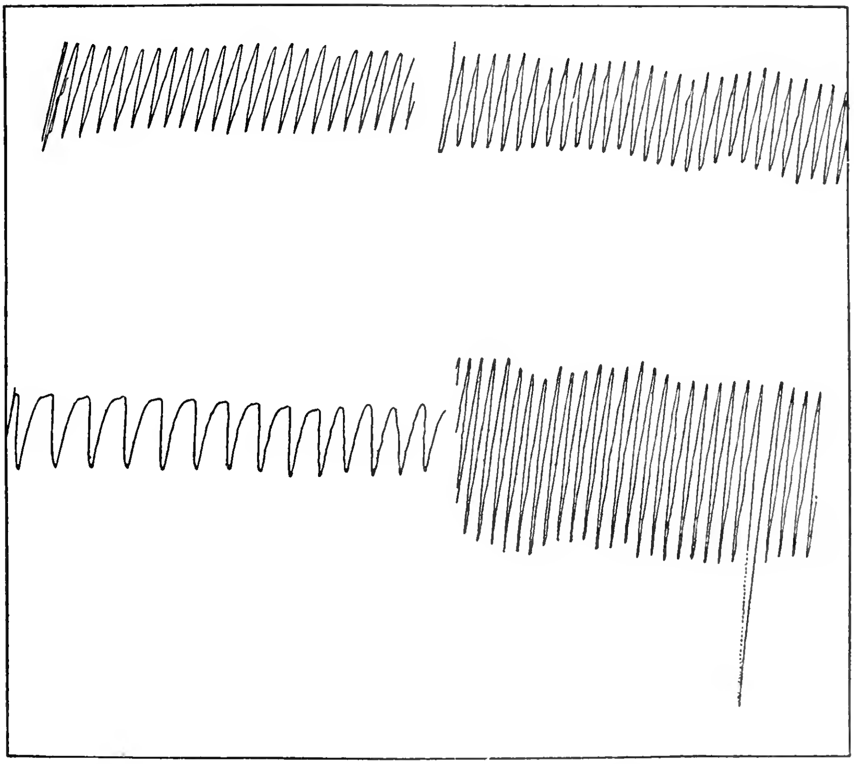
The observations upon dogs are given in Tables X and XI. They may be summarized as follows:

Carbon dioxide in the inspired air.	Increase in volume of tidal air in normal dogs.	Increase in volume of tidal air in pneumonic dogs. Temperature above 37.5°C.	Temperature 37.5°C. or less.
per cent	*per cent*	*per cent*	*per cent*
1	19	15	14
2	48	46	30
3	96	81	46
4	182	95	59
	285	127	68

These observations are shown graphically in Text-fig. 4. Text-fig. 5 reproduces the spirometer curves of a normal and a pneumonic dog.



TEXT-FIG. 4. The increase in tidal air in normal decerebrate dogs (uppermost curve), in pneumonic dogs with temperature above 37.5° C. (middle curve), and in pneumonic dogs with temperature 37.5° C. and below (lowest curve). The numbers at the left give the percentile increase in tidal air; the numbers below give the per cent of carbon dioxide. The data for these curves are the averages in Tables X and XI.



Text-Fig. 5. A comparison of the pneumonic and the normal respiratory reactions to carbon dioxide. The two upper curves show the tidal air at 1 and 5 per cent of carbon dioxide in Dog 14, Dec. 11, 1915, 5 hours before death from pneumonia. The two lower curves show the tidal air at 1 and 5 per cent of carbon dioxide in a normal decerebrate dog, Dec. 15, 1915.

It will be noted that the pneumonic dogs are divided into those moderately ill (rectal temperature above 37.5°C.) and those gravely ill (temperature 37.5°C. or less). The result of the disease is clearly seen by a comparison of the reactions of these groups when the inspired air contained 5 per cent of carbon dioxide.

Reaction at 5 per cent of carbon dioxide:

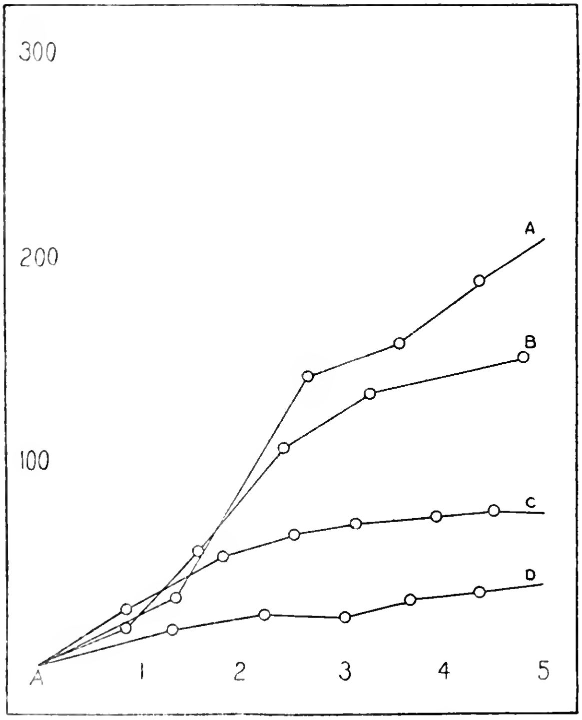
	per cent
In normal dogs	285
In dogs moderately ill with pneumonia	127
" " gravely ill with pneumonia	68

The progressive loss of the respiratory reaction in pneumonia is well shown by the observations made on Dog 9, 8, 4, 2, and $\frac{3}{4}$ hours before death.

Percentile increase in tidal air of Dog 9 as the carbon dioxide in the inspired air rose from 1 to 5 per cent.

Hrs. before death.	1 *per cent*	2 *per cent*	3 *per cent*	4 *per cent*	5 *per cent*
8	24	87	148	178	211
4	24	83	127	144	152
2	31	57	70	72	74
$\frac{3}{4}$	15	20	25	33	37

These observations are shown graphically in Text-fig. 6.



TEXT-FIG. 6. The reaction to carbon dioxide in Dog 9, Nov. 29, 1915, 8, 4, 2, and $\frac{3}{4}$ hours before death from pneumonia. The numbers at the left give the percentile increase in tidal air as the carbon dioxide inspired increases; the numbers below indicate the per cent of carbon dioxide. It is clear that the respiratory mechanism fails steadily.

The pneumonias thus far presented have been produced with *Bacillus pneumoniæ*, which is not often seen in clinical practice. The

ordinary pneumonia is caused by the pneumococcus. We find, however, no essential difference in the action of this organism—the reaction of the respiratory mechanism to carbon dioxide is lessened and finally abolished with the pneumococcus as with the bacillus of Friedländer. The following experiment is instructive.

On Apr. 12, 1916, a dog weighing 13.5 kilos received in the right bronchus 58 cc. of a liquid culture[10] of the pneumococcus. On Apr. 13 the rectal temperature at 9.30 a.m. was 39° C. The dog was tracheotomized at this time. At 9.45 a.m. the first carbon dioxide reaction was taken; the dog was able to walk about. At 3 p.m. the temperature was 39.9° C.; the dog could not maintain the sitting position; the second carbon dioxide reaction was measured. At 5 p.m. the temperature was 39.7° C.; the dog was semicomatose; the third carbon dioxide reaction was measured. At 6.20 p.m. the temperature was 39.7° C.; deep coma; corneal reflex present; hind legs becoming stiff; head slightly retracted; carbon dioxide reaction absent. At 6.30 p.m. the dog died. The autopsy showed typical red hepatization of the whole right lower, one-half the right middle, and one-half the right upper lobe.

The measurements in this dog were as follows:

Hrs. before death.	Percentile change in cc. breathed per min. as the carbon dioxide in the inspired air rose from 1 to 5 per cent. 1 *per cent*	2 *per cent*	3 *per cent*	4 *per cent*	5 *per cent*
$8\frac{3}{4}$	13	28	43	57	114
$3\frac{1}{2}$	7	18	40	100	171
$1\frac{1}{2}$	No reaction.			5	27
$\frac{1}{6}$	" "				

Although the course of the temperature differs in this animal from that usually observed with Friedländer's bacillus, there is no difference in the failure of the respiratory mechanism.[11]

CONCLUSIONS.

1. The reaction of the respiratory mechanism to carbon dioxide is greatly diminished in pneumonia.
2. The graver the disease, the less the reaction.

[10] The culture medium is glucose broth to which calcium carbonate has been added.

[11] We take pleasure in acknowledging our indebtedness to the industry and devotion of Mr. Luigi Freni, nine years an assistant in this laboratory.

THE INFLUENCE OF THE VAGUS NERVES UPON CONDUCTION BETWEEN AURICLES AND VENTRICLES IN THE DOG DURING AURICULAR FIBRILLATION.

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PLATE 43.

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The normal heart beat of the dog is affected differently by right and left vagus stimulation. Right vagus stimulation causes inhibition of impulse formation, slowing the rate of the heart beat or causing complete standstill. Left vagus stimulation has a less marked effect upon impulse formation, but seems to inhibit the conduction of impulses from auricles to ventricles more than right vagus stimulation. This difference was pointed out by Cohn,[1] who recorded the cardiac action by means of the string galvanometer. The writer[2] confirmed these observations in a series of experiments in which the same technique was employed. Although in these series of experiments it seemed clear that conduction between the auricles and ventricles was distinctly influenced more by the action of the left vagus, the comparison of the two nerves in this respect was confused by the predominant action of the right vagus in causing auricular slowing or stoppage. This difficulty has been pointed out by Cohn and Lewis,[3] who investigated the effect of the two nerves on conduction when auricular rate was maintained at a constant level by rhythmic induction shocks, at, or a little above threshold value, applied to the right auricle. They found that under these conditions profound

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[1] Cohn, A. E., On the Differences in the Effects of Stimulation of the Two Vagus Nerves on Rate and Conduction of the Dog's Heart, *J. Exp. Med.*, 1912, xvi, 732.

[2] Robinson, G. C., The Influence of the Vagus Nerves on the Faradized Auricles in the Dog's Heart, *J. Exp. Med.*, 1913, xvii, 429.

[3] Cohn, A. E., and Lewis, T., The Predominant Influence of the Left Vagus Nerve upon Conduction between the Auricles and Ventricles in the Dog, *J. Exp. Med.*, 1913, xviii, 739.

changes in conduction were obtained, which were generally more marked when the stimulus was applied to the left vagus than to the right.

The experiments to be reported in this paper are in some ways comparable to those of Cohn and Lewis, but instead of auricular impulses being maintained by rhythmic induction shocks, they were maintained by throwing the auricles into a state of constant activity by faradization. The result also is somewhat different. The type of auricular activity which results from faradization of the auricles is not true fibrillation alone, but consists in the presence of true fibrillary activity combined with auricular tachycardia involving large masses of the musculature.[2] For the purpose of the present paper, however, it seems unessential to draw a distinction between these two types of activity, and both will be spoken of as fibrillation.

The technique of the experiments has been described in a previous paper,[2] where the results of the effect of the stimulation of each nerve on the faradized auricles were reported, and will be referred to again only briefly. The electrocardiographic curves obtained in this series of experiments have been reviewed, especial attention being paid to two points. The first point is the relative effect of stimulation of the two nerves on conduction during auricular fibrillation. The second is the relative effect of left vagus stimulation in inhibiting the conduction of impulses set up by the normally beating auricles and in inhibiting the conduction of impulses set up by the fibrillating auricles. This comparison is possible in those experiments in which the normally beating auricles continued to beat during left vagus stimulation. By comparing the electrocardiographic records which showed this condition with those obtained during left vagus stimulation from the same animal while the auricles were fibrillating, it is possible to conclude whether impulses from the fibrillating auricles are more or less easily blocked by left vagus activity than normal auricular impulses.

No such comparison is possible with the right vagus, as a condition of auriculoventricular block was practically never produced in the normally beating heart with right vagus stimulation, auricular stoppage being a constant result.

EXPERIMENTAL.

In the series of twenty-seven experiments, thirteen allow a comparison of the effect of the two nerves on conduction during auricular fibrillation. Those experiments in which there was ventricular escape with auricular stoppage during the normal cardiac mechanism were discarded, for if ventricular escape should occur during vagus stimulation when the auricles were fibrillating, a false idea of the effect of vagus activity on conduction might be given.

Nine experiments allow a comparison of the effect of left vagus stimulation on conduction during the normal cardiac mechanism and during auricular fibrillation. In these experiments the auricular activity persisted when the left vagus was stimulated during the normal cardiac mechanism, and ventricular escape did not occur.

The experiments were carried on with the dogs under ether anesthesia. Electrodes were applied to the right fore leg and the left hind leg, and records were made with the Edelmann string galvanometer. The chest was opened and two clips to which stimulating wires were attached, were applied to the right auricle through a small opening in the pericardium. Each vagus was dissected out, and shield electrodes were applied. The auricles were faradized with varying strengths of currents, but the vagi were stimulated with a faradic current of constant strength, the secondary coil being usually pulled out 5 cm. In a few experiments a slightly weaker current was used. In some animals prolonged auricular fibrillation was produced by a single faradization, while in others frequent periods of faradization were necessary. This difference had no apparent effect on the action of the two nerves. The results of the experiments are given in tabular form, the details of all records which bear on the two points considered being shown by certain abbreviations.

In the first column is given the curve number, and in the second the type of auricular activity, N. B. signifying normal beat and A. F. auricular fibrillation. In the third column the duration of vagus stimulation in seconds is given, and in the fourth and fifth the effects of stimulation of the left and right nerves respectively are given. In describing the effects of vagus stimulation the term stoppage signifies arrest of the ventricles during auricular fibrillation, or of the whole heart during the normal beat. Complete block means that the auricles continued without ventricular responses; 2 : 1, 3 : 1, etc. express the ratio of auric-

ular and ventricular contractions, and the degree of partial heart block; 2 : 1^3 indicates the occurrence of three 2 : 1 cycles; P–R+ signifies a prolongation of the conduction interval. Figures in brackets indicate the length of time in seconds an effect has persisted after the termination of vagus stimulation. Other abbreviations have meanings that are self-evident.

The details of each experiment are given, followed by a tabulated summary of the results of all the experiments (Table I).

EXPERIMENT I.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
11	A. F.	4.2	Slowing, V. rate 102.	
15 A	" "	2.2		Stoppage, 2.6 sec. (0.4).
15 B	" "	2.4		" 3.0 " (0.6).
16	" "	4.3		" 4.4 " (0.1).

Summary.—Vagus action is uncontrolled on the normal beat. The right vagus is markedly more effectual in blocking impulses from the fibrillating auricles than the left vagus. The experiment is not conclusive, as there was but one left vagus stimulation.

EXPERIMENT II.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
9	A. F.	4.6		Stoppage, 1 beat in 5.0 sec.
14	" "	4.5	Stoppage, 2 beats in 6.2 sec.	
15	" "	5.0		" 2 beats " 5.0 "
16	" "	5.0	" 1 beat " 5.0 "	
18	" "	5.0	" 1 " " 7.6 "	
19	" "	5.0		" 1 beat " 6.2 "

Summary.—Vagus stimulation is uncontrolled on the normal beat. The right and left vagi are equally effectual in blocking impulses from the fibrillating auricles.

EXPERIMENT III.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
8	A. F.	5.6	Stoppage, 5.9 sec. (0.3). (Fig. 1).	
9	N. B.	4.6	Complete block, 4.6 sec. (0). A. rate, 110.5 (Fig. 2).	
10	A. F.	2.8		Stoppage, 3.2 sec. (0.4).
11	N. B.	3.9		" 3.9 " (0).

Summary.—The usual differences between stimulation of the right and left vagi are shown. The right and left vagi are equally effectual in blocking impulses from the fibrillating auricles. The left vagus stimulation caused blocking of impulses from the normally beating auricles as effectually as impulses from the fibrillating auricles.

EXPERIMENT IV.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
4	N. B.	5.4	P–R+ slight slowing.	
5	" "	5.5		Marked slowing, 3 beats in 5.6 sec.
8	A. F.	6.0	Slowing, 6 beats in 5.8 sec.	
10	N. B.	5.8	2 : 1[1], P–R+ slight slowing. Rate 120.	
11	" "	5.6		P–R slightly +, slowing.
14	A. F.	5.6		Slowing, 8 beats in 5.0 sec.
15	" "	6.0	Marked slowing, 2 beats in 3.6 sec.	
16	" "	6.4		Slight slowing, 14 beats in 5.6 sec.
17	" "	6.2	Marked slowing, 3 beats in 4.6 sec.	

Summary.—The usual differences between stimulation of the right and left vagi are shown, but they are not striking. The left vagus is more effectual than the right in blocking impulses from the fibrillating auricles, and is more effectual in blocking impulses from the fibrillating auricles than from the normally beating auricles.

EXPERIMENT V.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
12	N. B.	6.6	Complete block, 1.4 sec. P−R+. A. slight slowing.	
13	" "	6.4		Stoppage, 2.2 sec. Ectopic ventricular beats. A. much slowed.
16	A. F.	4.8	Stoppage, 1.9 sec. Slight slowing, 2.6 sec.	
17	" "	5.8		Stoppage, 5.6 sec. 4 ectopic ventricular beats.
18	" "	6.0		Stoppage, 5.6 sec. 4 ectopic ventricular beats.
19	" "	5.2	Stoppage, 2.1 sec. Slight slowing, 3.2 sec.	
20	" "	8.8	Stoppage, 2.2 sec. Slowing, 2.0 sec.	
21	" "	7.2		Stoppage, 6.8 sec. 5 ectopic ventricular beats.

Summary.—The effect of stimulation of the right and left vagus on the normally beating heart is confused by ectopic ventricular impulses. The right vagus is more effectual in blocking impulses from the fibrillating auricles than the left. The left vagus is slightly more effectual in blocking impulses from the fibrillating auricles than from the normally beating auricles.

EXPERIMENT VI.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
7	A. F.	5.7		Stoppage, 7.8 sec. (2.1).
8	" "	6.4	Stoppage, 8.8 sec. (2.4).	
9	" "	7.6		" 10.4 " (2.8).
10	" "	8.7	" 10.8 " (2.1).	
12	N. B.	4.0		" 5.2 " (1.2). P—R+ .2 beats.
13	" "	5.0	Complete block, 6.2 sec. (1.2). A. rate 92.	
14	A. F.	6.6	Stoppage, 7.4 sec. (0.8).	
15	" "	5.0		Stoppage, 7.0 sec. (2.0).
16	N. B.	3.7	Complete block, 4.5 sec. (0.8). A. rate 108.	
17	" "	5.0		" 6.2 " (1.2).
18	A. F.	5.8		" 8.0 " (2.2).
19	" "	6.9	Stoppage, 9.6 sec. (2.7).	

Summary.—The usual differences between stimulation of the right and left vagi are strikingly shown. The right and left vagi are equally effectual in blocking impulses from fibrillating auricles. The left vagus is as effectual in blocking the impulses from the normally beating auricles as those from the fibrillating auricles.

EXPERIMENT VII.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
5	A. F.	3.8		Stoppage, 6.0 sec. (2.2).
7	" "	5.2	Stoppage, 9.0 sec. (3.8).	
9	" "	6.8		" 9.4 " (2.6).
10	" "	6.0	" 9.0 " (3.0?).	
11	N. B.	6.7		" 9.2 " (2.5).
15	" "	3.0	" 5.0 " (2.0) $2:1^3$.	
16	" "	11.4		" 14.2 " (2.8).
17	" "	7.6	Stoppage, 8.6 sec. (1.0) $3:1^1$, $2:1^3$.	
18	A. F.	5.0	Stoppage, 7.6 sec. (2.6).	

Summary.—The usual differences between stimulation of the right and left vagi are shown, but they are not striking. The left vagus is slightly more effec-

tual than the right in blocking impulses from the fibrillating auricles, ventricular stoppage outlasting the period of left vagus stimulation longer than stimulation of the right. The effect of left vagus stimulation on the blocking of impulses from the normally beating auricles cannot be ascertained on account of stoppage of the auricles, which it caused.

EXPERIMENT VIII.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
5	A. F.	4.8	Marked slowing, V. rate 53.	
12	" "	5.4		Marked slowing, V. rate 73.

Summary.—A comparison of the effect of stimulation of the two nerves on the normally beating heart was not obtained. The left nerve is more effectual in blocking impulses from the fibrillating auricles than the right.

EXPERIMENT IX.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
6	N. B.	1.8		Stoppage, 2.2 sec. (0.4).
7	" "	3.8	Complete block, 4.0 sec. (0.2). A. rate 120.	
9	" "	3.2		Stoppage, 2.2 sec.; then slowing. P—R slightly+.
10	" "	4.0	$3:1^1, 2:1^3$.	
13	" "	6.6	$6:1^1, 2:1^5$.	
14	" "	3.2		Stoppage, 2.2 sec.; then slowing. P—R not +.
16	A. F.	4.4	Stoppage, 2.2 sec.; then marked slowing. Rate 45.	
17	" "	7.8		Stoppage, 0.8 sec.; then slowing. Rate 123.
18	" "	6.0	Stoppage, 3.3 sec. 2 beats in 6.2 sec.	
20	" "	5.8		Slowing, 6 beats in 5.6 sec.

Summary.—The usual differences between stimulation of the right and left vagi are strikingly shown. The left vagus is more effectual in blocking impulses from the fibrillating auricles than the right, while it causes blocking of impulses

from the normally beating auricles and from fibrillating auricles in an equal degree.

Curves 13, 14, 16, and 17 have been reproduced in a previous paper[2] as figures 4, 3, 6, and 5 respectively.

EXPERIMENT X.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.*	Right vagus effects.
		sec.		
4	N. B.	3.0		Stoppage, 3.0 sec. (0) $2:1^1$.
5	" "	5.1	$3:1^1$, $2:1^3$. P−R+. A. rate 103.	
8	A. F.	4.6		" 5.6 sec. (1.0).
10	" "	6.1	Stoppage, 3.0 sec. 3 beats in 6.6 sec.	
11	" "	5.4		" 5.4 " (0).
14	N. B.	6.2	$2:1^5$. P−R+. A. rate 58.	
15	" "	3.0		" 3.0 " (0). $2:1^1$, P−R+.
20	" "	4.1	Stoppage, 3.6 sec. $5:1^1$, $2:1^1$. A. rate 80.	
21	A. F.	3.0	Stoppage, 3.2 sec. (0.2).	

* Nerves stimulated with secondary coil out 6 cm.

Summary.—The usual differences between stimulation of the right and left vagi are strikingly shown. Right and left vagi are equally effectual in blocking impulses from the fibrillating auricles. The left vagus is as effectual in blocking impulses from the normally beating auricles as those from the fibrillating auricles.

EXPERIMENT XI.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.*	Right vagus effects.
		sec.		
4	N. B	4.2		Stoppage, 6.4 sec. (1.8).
5	" "	4.8	Complete block, 5.2 sec. (0.4). A. rate 116.	
6	" "	2.3		" 4.0 " (1.7).
7	" "	3.7	Complete block, 4.4 sec. (0.7). A. rate 100.	
8	A. F.	4.0		" 4.4 " (0.4).
9	" "	5.1	Stoppage, 4.8 sec.	
11	" "	6.3	" 6.6 " (0.3).	
20	N. B.	5.4	Complete block, 3.0 sec. A. rate 117.	
21	" "	1.4		" 4.4 " (3.0).
22	A. F.	4.4	Stoppage, 4.2 sec.	
23	" "	4.9		" 6.2 " (1.7).

* Nerves stimulated with secondary coil out 7 cm.

Summary.—The usual differences between stimulation of the right and left vagi are strikingly shown. The right vagus is slightly more effectual in blocking impulses from the fibrillating auricles than the left, as shown by the fact that ventricular stoppage always outlasts the periods of right vagus stimulation, while it usually does not outlast the periods of left vagus stimulation. The left vagus is as effectual in blocking the impulses from the normally beating auricles as those from the fibrillating auricles.

EXPERIMENT XII.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
9	A. F.	4.8	Marked slowing, 6 beats in 4.8 sec.	
10	" "	5.4		Slight slowing.
11	N. B.	5.6	$2:1^3$. P—R++. 3 V. beats in 2.6 sec.	
12	" "	7.0		No effect.
13	" "	4.4	$2:1^3$. P—R++. 3 V. beats in 2.6 sec.	
14	" "	8.4		" "
17	" "	4.2		Stoppage, 1.6 sec.; then marked slowing.
18	" "	5.2	$2:1^5$. P—R++. 6 V. beats in 4.4 sec.	
26	A. F.	6 6		No effect.

Summary.—The right vagus is not effectual. The left vagus is as effectual in blocking impulses from the normally beating auricles as those from the fibrillating auricles.

EXPERIMENT XIII.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
3	N. B.	5.0		Stoppage, 4.0 sec. P—R+.
4	" "	5.0	Stoppage, 4.6 sec. 1 blocked A. beat, P—R+.	
5	" "	3.2		" 3.6 " (0.4). P—R+.
6	" "	3.4	Stoppage, 3.2 sec. 1 blocked A. beat, P—R+.	
9	A. F.	5.0		Stoppage, 5.6 sec. (0.6).
10	" "	5.4	Stoppage, 5.6 sec. (0.2).	
11	" "	5.0		" 6.0 " (1.0).
12	" "	6.2	" 6.0 "	
13	N. B.	5.2		" 5.6 " (0.4). 2 blocked A. beats. P—R+.
14	" "	5.4	" 5.4 " P—R+.	
16	" "	4.0		Stoppage, 5.6 sec. (1.6). P—R not +.
17	" "	4.0	" 4.2 " 1 blocked A. beat.	
20	A. F.	5.6		Stoppage, 6.6 sec. (1.0).
22	" "	3.2	Stoppage, 3.2 sec.	
23	" "	5.0		" 5.8 " (0.8).

Summary.—The usual differences between stimulation of the right and left vagi are shown, but they are only slight. The right vagus is slightly more effectual in blocking impulses from the fibrillating auricles than the left, as is indicated by the fact that blocking always outlasts the periods of right vagus stimulation, while it usually does not outlast the periods of left vagus stimulation. The effect of left vagus stimulation on blocking of impulses from the normally beating auricles cannot be ascertained on account of the stoppage of auricles which it causes.

EXPERIMENT XIV.

Curve No.	Auricular activity.	Duration of vagus stimulation.	Left vagus effects.	Right vagus effects.
		sec.		
3	N. B.	3.6		Stoppage, 4.0 sec. (0.4).
4	" "	7.8	$3:1^1$, $2:1^8$. 9 V. beats in 7 sec. A. rate 165. V. rate 80.	
7	A. F.	4.3		" 3.8 "
8	" "	5.4	Slowing. 7 beats in 4.2 sec. V. rate 109.	

Summary.—The usual differences between stimulation of the right and left vagi are strikingly shown. The right vagus is strikingly more effectual than the left in blocking impulses from the fibrillating auricles. The left vagus is nearly or entirely as effectual in blocking impulses from the fibrillating auricles as those from the normally beating auricles.

The results of these experiments are given in tabular form (Table I). In the third column are given the effects of stimulation of the right and left vagi on the normally beating heart. These effects serve as a control for the activity of the nerves during auricular fibrillation, except in three experiments in which the auricles were always faradized before the vagi were stimulated. When the effects are described as "usual," it signifies that the right nerve was more effectual in causing stoppage of the whole heart, while the left nerve was apparently more effectual in causing blockage of the impulses from the auricles.

The effect that stimulation of each nerve had on conduction during auricular fibrillation has been estimated by the degree of ventricular slowing which took place or the length of time ventricular stoppage occurred relative to the duration of vagus stimulation. These results are given in the fourth double column. In the last double column are given the effects which left vagus stimulation had on the ventricular rate during auricular fibrillation and during the normal beat in those experiments in which normal auricular activity persisted during left vagus stimulation.

TABLE I.

Tabulated Results of Experiments.

Experiment No.	Series No.	Effects on normal beats.	Effect on conduction during A.F.		Effect of left nerve on conduction.	
			Right.	Left.	A.F.	N.B.
I	103	Not recorded.	+	–	Not determined.	
II	105	" "	Equal.	Equal.	" "	
III	108	Usual, marked differences.	"	"	Equal.	Equal.
IV	111	" slight "	–	+	+	–
V	112	Undetermined.	+	–	+	–
VI	113	Usual, marked differences.	Equal.	Equal.	Equal.	Equal.
VII	114	" slight "	–	+ slight.	Not determined.	
VIII	115	Not recorded.	–	+	" "	
IX	118	Usual, marked differences.	–	+	Equal.	Equal.
X	121	" moderate "	Equal.	Equal.	"	"
XI	122	" marked "	+ slight.	–	"	"
XII	125	Right nerve ineffectual.	Not determined.		"	"
XIII	126	Usual, slight differences.	+ slight.	–	Not determined.	
XIV	127	" marked "	+	–	Equal.	Equal.
Totals 14.		9 characteristic. 5 undetermined.	5+	4+	2+	0+
			4 equal. 1 undetermined.		7 equal. 5 undetermined.	

DISCUSSION.

The thirteen experiments in which the effect of right and left vagus stimulation on ventricular activity during auricular fibrillation was compared indicate that there is no constant difference. The right nerve was more effectual in slowing or stopping the ventricles in five experiments, the left nerve was more effectual in four, while in four experiments the two nerves were equally effectual. Impulses apparently continue to be sent down frequently toward the ventricles. The fact that ventricular activity is strikingly inhibited by vagus stimulation under either condition indicates, therefore, that the impulses from the auricles are blocked. The degree to which the ventricular activity is diminished or the duration of ventricular stoppage relative to the duration of vagus stimulation may be taken as the measure of the effect of vagus stimulation on conduction between auricles and ventricles.

The experiments fail to show any constant difference in the effect which stimulation of the right and of the left vagus nerves has on conduction between auricles and ventricles of the dog's heart when auricular fibrillation is present. These results are not in accord with those of Cohn and Lewis, who found a difference in the influence of the two nerves on auriculoventricular conduction when the auricular activity was maintained during vagus stimulation by rhythmic electric stimulation. It is possible that this disagreement is to be found in the difference in auricular activity. In a previous paper[2] it was pointed out that the auricular activity following faradization is differently altered by stimulation of the two nerves. Stimulation of the right nerve causes a type of auricular activity in which the separate muscle fibers become more independently active, while stimulation of the left nerve causes the auricular activity to be of a coarser type, more nearly approaching auricular tachycardia.

The difference in auricular activity does not seem to be sufficient to explain the differences between the results of the experiments reported here and those of Cohn and Lewis. The reason for the discrepancy is obscure.

The nine experiments which afford an opportunity for comparing the effect of left vagus stimulation on conduction during the normal beat and during auricular fibrillation show that the type of auricular activity has apparently no influence on the effect which stimulation of the left vagus has on auriculoventricular conduction. With two exceptions the ventricular stoppage or slowing caused by the stimulation when the auricles were fibrillating was identical with that obtained during the normal heart beat. In only one experiment was left vagus stimulation definitely more effectual in blocking impulses from the fibrillating auricles than from the normally beating auricles. These results indicate that the type of auricular activity has no influence on the degree to which impulses are blocked by vagus stimulation. This fact is of interest as bearing upon the action of digitalis in auricular fibrillation. It has been suggested that digitalis is especially potent in blocking impulses sent to the ventricles from fibrillating auricles. In the light of these experiments it would seem that the character of the auricular activity whether coordinate or fibrillatory plays no part in the effectiveness of the drug.

CONCLUSIONS.

The experiments that have been reported indicate that stimulation of either the right vagus or the left vagus nerve is equally effectual in blocking impulses from the auricles to the ventricles when auricular fibrillation is present. Stimulation of the left vagus nerve is as effectual in blocking impulses from the normally beating auricles as from the auricles when in a state of fibrillation, and the type of auricular activity has apparently no influence on the effect which stimulation of the left vagus has on auriculoventricular conduction.

EXPLANATION OF PLATES.

Plate 43.

Fig. 1. Experiment III. The effect of left vagus stimulation during auricular fibrillation. Ventricular stoppage for 5.9 seconds. Stimulation of 5.6 seconds' duration.

Fig. 2. Experiment III. The effect of left vagus stimulation during normal auricular activity. Ventricular stoppage for 4.6 seconds. Stimulation of 4.6 seconds' duration.

THE INTERRELATION OF THE SURVIVING HEART AND PANCREAS OF THE DOG IN SUGAR METABOLISM.

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The problem of sugar metabolism and diabetes is intimately associated with the two following questions: (1) Does the pancreas have a local independent action on dextrose passing through its blood vessels, altering the dextrose in some way for utilization by the body tissues? (2) Is there a direct interrelation between the pancreas and body tissues in dextrose metabolism, and if so, how is it accomplished?

In the experimental work reported below the attempt has been made to gain some evidence as to the first of these questions by the direct perfusion of the pancreas with physiological concentrations of dextrose. The second question has been studied by various types of perfusion experiments on the isolated surviving heart and pancreas in order to determine whether there was any interdependence between the two in dextrose metabolism.

The numerous experiments recorded by various workers bearing upon the interrelation between the pancreas and body tissues in the utilization of dextrose need not be reviewed in detail. It is sufficient to say that two general lines of investigation have developed. On the one hand, the experiments of Hédon (1), Carlson and Ginsburg (2), Forschbach (3), Carlson and Drennan (4), Verzár and von Fejér (5), Woodyatt (6), Murlin and Kramer (7), and others, in which blood was transferred in various ways from normal to diabetic animals in the hope of thus supplying a pancreatic hormone, have yielded no decisive results. Again, Verzár and von Fejér (5), Murlin and Kramer (7), Scott (8), and others have attempted to supply this hypothetical internal secretion by the injection into diabetic animals of various kinds of pancreatic extracts. Their results have in general been negative. The obvious objection can be raised to all such experiments that in grinding the pancreas its powerful proteolytic enzymes might readily inactivate a more delicate product of internal secretion.

TABLE I.

Summary of Experiments on the Utilization of Dextrose by the Isolated Mammalian Heart.

Author	Date.	Type of heart.	No. of experiments.	Circulating medium.	Strength of dextrose perfused.	No. of hrs. perfused.	Interval between sugar determinations.	Dextrose disappeared per gm. of heart per hr.	Bacterial counts.	Final perfusate hydrolyzed	Aseptic precautions. Special notes.
					per cent			*mg.*			
J. Müller (17).	1904	Cat.	4	Ringer-Locke's.	0.1 ±	2-6	At end of experiment.	2 ±	None.	No.	Not mentioned. Heart estimated as 12 gm.
Locke and Rosenheim (18)	1907	Rabbit.	4 beating. 3 not beating.	"	0.1–0.25	7–10	At beginning and end of experiment.	11.2–1.7	"	Yes.	Not mentioned.
Camis (19)....	1908	" Cat.	20 9	"	0.1 ±	25–69 min.	At end of experiment.	0 13	"	No.	Not mentioned. Cat's hearts estimated as 12 gm. Rabbit's hearts estimated as 8 gm.
Rohde (20)...	1910	"	12	"	0.2	4 ±	At 2nd and 4th hrs.	0.98 1st. 1.64 2nd.	"	"	Apparatus washed with alcohol. Aseptic surgical technique not mentioned.
Gayda (21)...	1912	Rabbit.	10	"	0.1	4–6	At end of experiment.	3.8–14.9 Av. 7.1	"	"	Apparatus washed with alcohol. Aseptic surgical technique not mentioned.
Knowlton and Starling (22)	1912	Dog.	10 normal. 7 diabetic.	Heart-lung preparation.	0.2–0.89	1–3	1 hr. ±	Normal 4 Diabetic 0	"	"	Attempt at sterilization of apparatus in a few experiments. Aseptic surgical technique not mentioned.
Neukirch and Rona (23)...	1912	Cat.	Glucose 1.	Ringer-Locke's.	1.0	$6\frac{1}{2}$	At end of experiment.	1.6	"	"	Apparatus sterilized with concentrated acid and water. Aseptic surgical technique not mentioned.

Maclean and Smedley (24)	1913	Rabbit. Dog.	4 3	Ringer-Locke's.	0.1–0.2	1–4	Successive hrs.	0.7 (av.) 1.3 (av.)	None.	No.	Apparatus sterilized in autoclave. "Ordinary aseptic precautions."
Maclean and Smedley (25)	1913	Diabetic dog.	12	"	0.1–0.3	3		0 +	"	"	Apparatus sterilized in autoclave. "Ordinary aseptic precautions."
Mansfeld (26).	1913	Rabbit.	5 normal. 8 fever.	"	0.1	3	At $1\frac{1}{2}$ and 3 hrs.	Normal 2.2 Fever 4.4	"	"	Not mentioned.
Patterson and Starling (27)	1913	Dog.	9 normal. 14 diabetic.	Heart-lung preparation.	0.2–0.7	1–3 2	At end of experiment.	Normal 0+ Diabetic 0+	"	"	Apparatus washed with soda and occasionally boiled. Aseptic surgical technique not mentioned.
Cruickshank (28)	1913	"	6 normal. 13 diabetic.	Heart-lung preparation.	0.4–0.6	1–4 $1\frac{1}{2}$–4			"		Same as preceding experiment. Experiments conducted to study glycogen content of hearts.
Cruickshank and Patterson (29)....	1913	Cat. Dog.	14 8 diabetic.	Ringer-Locke's.	0.2–0.3	1–3	Perfusions divided into two periods.	1.5 0.9	"	Yes.	Apparatus washed with boiling water. Aseptic surgical technique not mentioned.
Starling and Evans (30)..	1914	"	Numerous.	Heart-lung preparation.	?	Varying.		Estimated from RQ. 1.6	"	No.	Not mentioned.
Underhill and Prince (31)..	1914	Rabbit.	25	Ringer-Locke's.	0.1	2	At end of experiment.	0.7–3.8	"	"	Apparatus sterilized in autoclave. State that aseptic precautions were observed.

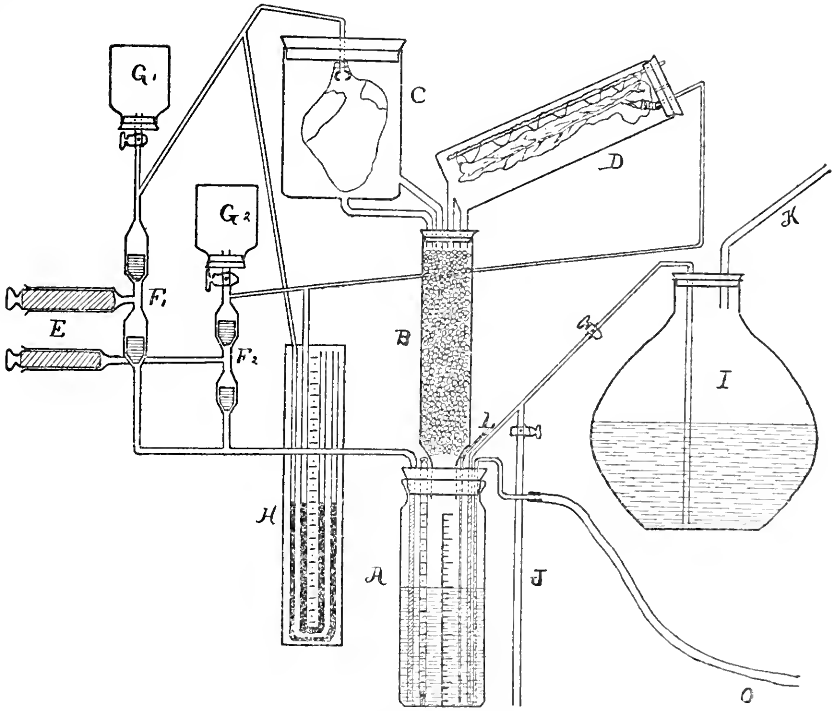
The second main line of investigation has developed since Cohnheim (9) in 1903 reported experiments in which he found that while pancreatic extract or muscle extract alone had no effect on dextrose, a mixture of the two caused a rapid disappearance of sugar, which he interpreted as being due to an oxidative process. Although his results were attacked by Claus and Embden (10) and others, they were supported by Arnheim and Rosenbaum (11), Dewitt (12), and Hall (13), and were generally accepted until Levene and Meyer (14) in 1911 found that this apparent destruction of dextrose was due to a condensation process, for by hydrolysis the reducing sugar was restored.

Method.

The method of isolated perfusion was chosen in the following experiments, for, while obviously artificial compared to normal physiological conditions, it offered a method of controlling the factors concerned.

Two references only to the direct perfusion of the pancreas have been found. Hustin (15) perfused the pancreas in a study of its external secretion, and de Meyer (16) in 1910 found that by adding a solution previously perfused through a pancreas to one subsequently perfused through a liver there was an increase in the liver glycogen. The findings of fifteen observers who have studied the utilization of sugar by the isolated mammalian heart are summarized in Table I. An analysis of their results brings out the following criticisms.

1. A uniform type of animal has not been used.
2. The conclusions of the different observers are based on no constant time period of perfusion, but vary from 25 minutes to 10 hours.
3. Save for the experiments of Maclean and Smedley (24), there has been no attempt to make hourly sugar determinations in order to follow the rate of sugar disappearance.
4. There has been a great discrepancy between the findings of various workers as to the amount of dextrose used by the living heart. Patterson and Starling (27) in the most recent work using a heart-lung preparation and correcting for the sugar destroyed in the lung found very little if any sugar used either by a normal or diabetic heart in periods of 1 to 3 hours.
5. Locke and Rosenheim (18) and Cruickshank and Patterson (29) are the only workers who hydrolyzed the final perfusates to determine whether by so doing there was an increase in the reducing substance present.
6. The observance of aseptic precautions has varied greatly with different workers. Maclean and Smedley (25) and Underhill and Prince (31) alone state that their apparatus was sterilized in an autoclave. In no instance save in the experiments of Underhill and Prince is there mention of scrupulous, aseptic, surgical technique, and in no instance are bacterial counts given.



TEXT-FIG. 1. Diagram of the perfusion apparatus. Reduced to about one-sixth.

A. Reservoir containing the perfusion fluid, consisting of a 400 cc. graduated glass bottle with a rubber stopper through which the various tubes and thermometer pass.

B. Artificial lung consisting ot a glass cylinder filled with glass beads.

C. Glass perfusion chamber in which the heart is suspended from the bulb-shaped cannula tied in the aorta. The cannula passes through the large rubber cork. The fluid after perfusing through the coronary arteries flows from the auricles and passes down through the lung into the reservoir.

D. Glass perfusion chamber in which the pancreas is suspended by its mesenteric border to the long glass arm which projects from the rubber cork. The cork is perforated by an opening for the escape of oxygen and one for transmitting the cannula to the pancreatic artery. The perfused fluid drips from the pancreas and flows down through the lung to the reservoir.

E. 3 and 5 cc. Luer syringes acting as pumps, and operated by a motor attachment.

F_1 and F_2. Glass two-way valves.

G_1 and G_2. Glass bottles acting as air-heads.

H. Double manometer connected with each of the delivery systems.

I. Reserve reservoir with syphon connection to the perfusion reservoir.

J. Tube for draining the perfusion reservoir.

K. Blow-tube for starting the syphon.

L. Small tube for removing samples of perfusate for analysis.

O. Oxygen tube. The oxygen bubbles through the reservoir and passes up through the column of beads against the stream of perfusate coming down from the organs.

The importance of this last criticism has been emphasized by the results of this investigation. Infection of the perfusing liquid occurs easily and its occurrence may account for a slight or even a considerable disappearance of dextrose. Extreme care in sterilization of the apparatus and the observance of strict aseptic precautions are necessary to exclude this factor. All my final experiments were controlled by bacterial counts, and those in which the precautions against infection were not successful, as shown by the bacterial counts, were discarded. It seems evident that previous workers have failed to realize that not only must ordinary aseptic precautions be used, but that only by the minute observance of the most rigid care controlled by bacterial counts can bacteria be sufficiently excluded to make the conclusions as to sugar utilization in such experiments reliable.

Apparatus.—The diagram of the apparatus used is shown and explained in Text-fig. 1. Extreme care was taken to wash all adherent fat and protein from the apparatus immediately at the end of each experiment. Sterilization was accomplished by fractional sterilization in an Arnold sterilizer. Immediately before each experiment the apparatus was tested by perfusing several times with sterile Locke's solution containing the percentage of dextrose to be used in the experiment. This technique was time-consuming but was justified by repeatedly getting an apparatus, washings from which showed no bacteria on agar plates. The temperature of the apparatus and perfusate in all experiments was maintained at 37–38.5°C. by keeping the apparatus in a thermostat.

Perfusate.—The solution used in all experiments was a standard Ringer-Locke's solution of the following formula: calcium chloride 0.024 per cent, potassium chloride 0.042 per cent, sodium bicarbonate 0.03 per cent, sodium chloride 0.9 per cent, dextrose (Kahlbaum's pure) 0–0.5 per cent (according to the experiment). Locke's solution was made up immediately before each experiment from freshly distilled water which was sterilized by boiling. The amount of perfusate was generally 250 cc. and was left unchanged during the period of perfusion, save for samples removed for analysis.

Dextrose Determinations.—A slight modification of the colorimetric method of blood sugar determination described by Lewis and Benedict (32) was used. The great advantage which the method offered was

that the small amount of fluid necessary for analysis did not materially diminish the volume of the perfusate, so that hourly observations were possible. The results reported in these experiments obviously depend on the accuracy of the technique of sugar determination. For this reason in the earlier experiments hourly duplicate determinations were made not only on the perfusate but on the control perfusion and on the original Locke's solution. The average error of duplicate determinations practically never exceeded 0.5 per cent of the total sugar present. Correction was always made for the amount of perfusate removed for analysis in estimating the total volume of sugar present in the perfusate.

Controls.—A small apparatus similar in all respects to the main apparatus save that it contained no living tissue was used in the first forty-seven experiments. Its reservoir of 50 cc. was filled from the main apparatus after the heart or pancreas or both had been perfused for 10 minutes. Thus the control apparatus contained a fluid which was inoculated with whatever bacteria were contained in the main perfusate and with whatever enzymes were washed out in that time. In no experiment considered was there any change in the reducing properties of the sugar in the control perfusion beyond the limits of experimental error.

Bacterial Counts.—The plate method of bacterial counting was used throughout. Contaminating organisms were usually of the intestinal types. While contamination generally occurred even with the most rigid precautions, it was possible to estimate from the number of bacteria present whether they were sufficient to account for a disappearance of sugar. Six experiments were obtained which remained entirely sterile for the 4 hour period of perfusion. Those experiments were discarded in which bacterial contamination might in any way be interpreted as having accounted for dextrose utilization.

Hydrolysis.—The method of hydrolysis used by Levene and Meyer (14) was followed: hydrolyzing for 2 hours with 1 per cent hydrochloric acid with a reflux condenser, and subsequently carefully neutralizing and bringing to the original volume. It was found that this percentage of acid gave the maximum hydrolytic effect. If a polymerization of the dextrose into some more complex, non-reducing form occurred, hydrolysis should restore it to the simple mono-

saccharide. In this way it is possible to determine whether the disappearance of dextrose is a real utilization or only an apparent one.

Surgical Procedure.—Small or medium sized dogs were used which had been previously fed on a routine meat-bread diet. Great care was observed in shaving and sterilizing the chest and abdomen of each dog. All instruments were sterilized by boiling. Rubber gloves and sterile gowns were worn by the operator and his assistant. The table and animal, except the narrow field of operation, were covered with sterile towels. In a word, every precaution of a major surgical operation was used. The dogs were anesthetized with ether.

Removal of the Pancreas.—The removal and perfusion of the pancreas is a simple operation. The abdomen was opened by a transverse incision just below the costal margin. The aorta was clamped as it emerged from the diaphragm thus making the subsequent steps practically bloodless. The pancreaticoduodenal artery was exposed and a cannula placed in it. All the pancreas except the splenic portion which is not supplied by the pancreaticoduodenal artery was then gently removed. The common bile-duct must not be cut. The pancreas was then washed in warm sterile Locke's solution and loosely suspended by a continuous suture through its mesenteric border to the supporting arm of the perfusion chamber and placed in the apparatus, as shown in Text-fig. 1. The cannula was connected and the perfusion started. The procedure can be carried out in less than 5 minutes. A pulsatile pressure whose systolic pressure never exceeded 40 mm. of mercury was used, and about 20 cc. of fluid were perfused per minute. Carmine gelatin injections of the pancreas by this method showed that there was complete penetration of the fluid to every portion of the pancreas.

Removal of the Heart.—The technique of removal and perfusion of the heart scarcely need be described as it was similar to that used by previous workers. The thorax was rapidly opened, the heart removed and transferred to a dish of warm sterile Locke's solution, where, after washing it free of any excess of blood, the cannula was tied into the aorta above the aortic valves. Care must be used to exclude air bubbles. The heart was placed in the perfusion chamber and perfusion begun at once. This procedure requires about 3 min-

utes. The heart generally beats vigorously from the first. At the end of 4 hours the heart always showed a varying amount of edema and dilatation. As soon as the hearts were allowed to die, however, and rigor mortis set in, a considerable amount of this fluid seemed to be squeezed out. In addition every heart was mechanically squeezed free of as much fluid as possible. From gross appearance the heart approximated its original size. The error that resulted from this procedure must have been slight, and fairly uniform for all the experiments. In the last thirteen experiments the hearts were weighed before and after perfusion and the results justified the previous method.

The amount of fluid perfused through the hearts varied from 20 to 60 cc. per minute according to the size of the heart. To force this amount of fluid through the coronary arteries required a pulsating pressure whose systolic pressure varied from 40 to 160 mm. of mercury, and the diastolic pressure from 20 to 100 mm. What factor caused this variation in the resistance of the coronary arteries has not been determined. In all experiments recorded, unless otherwise noted, the hearts continued to beat for the 4 hour period.

Perfusion of the Heart and Pancreas.—In a simultaneous perfusion of the heart and pancreas from the same animal the heart was first removed and then the pancreas, as described above. The double perfusion is shown in Text-fig. 1. It is obvious that the intimate mixture of the fluids passing through the two organs was practically equivalent to a perfusion of the organs in series. If the pancreas was to be perfused first it was removed and the dog was kept under light ether anesthesia until the heart was required.

RESULTS.

Pancreas Perfusions.—Eleven experiments were carried out before the technique of perfusion was perfected. In these experiments there was a distinct disappearance of dextrose from solutions perfused through the pancreas. This might have been interpreted as a utilization of, or change in the dextrose, had bacterial counts not been made. Table II is given to illustrate these experiments, and shows that at the end of 5 minutes the perfusate contained about 350,000 bacteria per cc. In the first 3 hours there was a slight drop in the

TABLE II.

Infected Pancreatic Perfusion.

Fluid.	Sugar, 5 min.	Sugar, 3 hrs.	Sugar, 6 hrs.	Bacteria per cc. at end of 5 min.	Bacteria per cc. at end of 6 hrs.
	per cent	*per cent*	*per cent*		
Original Locke's solution......	0.3041	0.3030	0.3035	0	0
Control perfusion.............	0.3041	0.3058	0.3041	350,000	19,400,000
Pancreatic perfusion..........	0 3026	0.300	0 2347	350,000	50,500,000

percentage of dextrose. In the subsequent 3 hours there was a considerable loss of sugar but the bacterial counts at the end of this 6 hour perfusion showed over 50,000,000 bacteria per cc. With perfection of the technique, however, three successive perfusions of the pancreas were obtained in which the maximal bacterial count was 1,670,000. In none of these experiments was there the least detectable loss of dextrose. Table III shows the summarized results of these experiments. In these experiments it seems evident that when

TABLE III.

Pancreas Perfusion.

Experiment No.	5 min.		1 hr		2 hrs.		3 hrs.	
	Dextrose in perfusate.	Dextrose in control.	Dextrose in perfusate.	Dextrose in control.	Dextrose in perfusate.	Dextrose in control.	Dextrose in perfusate.	Dextrose in control.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
22	0.298	0.298	0.300	0.299	0.298	0.299	0.300	0.300
23........	0.601	0.601	0.599	0.597	0.601	0.600	0.599	0.601
24	0.10	0.10	0.099	0.099	0.100	0.099	0.10	0.10

Experiment No.	4 hrs.		5 hrs.		6 hrs.		Bacteria.		Dextrose after hydrolysis.	Dextrose disappeared per gm. of pancreas.
	Dextrose in perfusate.	Dextrose in control.	Dextrose in perfusate.	Dextrose in control.	Dextrose in perfusate.	Dextrose in control.	5 min.	6 hrs.		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*	
22	0.298	0.299	0.298	0.299	0.300	0.300	40	22,560	0.296	0
23	0.600	0.600	0.600	0.600	0.601	0.599	12	295,000	0.599	0
24	0.099	0.10	0.099	0.099	0.099	0.10	101	1,670,000	0.099	0

dextrose is perfused through the pancreas there is neither a change of the dextrose into a non-reducing form, nor does hydrolysis alter its reducing properties.

Heart Perfusions.—In view of the variations reported by different workers as to the amount of dextrose utilized by the surviving heart, and in view of the obvious criticisms stated above, it was felt that before proceeding to perfuse the heart and pancreas together it would be necessary to determine the utilization of dextrose by the heart alone under the conditions used in these experiments. Five successful perfusions of the heart were obtained over a period of 4 hours in which the maximal bacterial count was 216,000 per cc. Table IV is a condensed chart of these experiments, and they are shown in an average curve in Text-fig. 2. The experiments indicate that during the 1st hour, in averaging the five experiments, the sugar utilization was 0.028 mg. per gm. of heart, in the 2nd hour 0.013 mg., in the 3rd hour 0.242 mg., and in the 4th 0.415 mg. It was felt that this disappearance of sugar in the last 2 hours was not due to bacterial action, for not only was there no loss of dextrose from the control perfusions, but in the perfusions of the pancreas for periods of 6 hours in which the maximal bacterial count exceeded any of those in the heart perfusions there was no loss of dextrose. In these experiments when the final perfusate was hydrolyzed there was no definite increase in reducing substance.

Heart-Pancreas Perfusions.—Six experiments were obtained in which the heart and pancreas were simultaneously perfused with the same fluid and in which the maximal bacterial count at the end of 4 hours was about 800,000 per cc. In two of these experiments, as will be seen by consulting Table V, the perfusing liquid remained practically sterile throughout. In Experiment 32 the heart suddenly stopped at the end of about $2\frac{1}{2}$ hours. The figures, however, are given. The results are diagrammatically shown in Text-fig. 2 and summarized in Table VI.

As the experiments progressed it became apparent, as shown in Text-fig. 2, that a striking phenomenon was occurring. The disappearance of sugar began in the 1st hour and was greatly in excess of that occurring when the heart was perfused alone. In the heart perfusions the distinct disappearance of sugar begins late, after the

TABLE IV.

Condensed Table of Experiments in Which the Heart Was Perfused Alone.

Experiment No.	Weight of heart.	Dextrose after 10 min. perfusion.	1st hr.			2nd hr.			3rd hr.			4th hr.			Dextrose per gm. of heart recovered by hydrolysis.	Average error of duplicate determinations.	Dextrose disappeared from control perfusion.
			Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.			
	gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*	*per cent*	
26......	84	0.200	0.201	0	85	0.199	0.03	83	0.200	0	1,888	0.192	0.237	3,500		0.66	0
27......	65	0.300	0.299	0.011	608	0.299	0	9,920	0.299	0		0.273	0.91			0.25	0
28......	46	0.368	0.368	0.018	190	0.367	0.036	258	0.366	0.043	53,200	0.3655	0.025	216,000	0	0.12	0
29......	69	0.501	0.497	0.11	44	0.499	0	7,100	0.473	0.933	7,800	0.456	0.678		0	0.65	0
34......	82	0.099	0.100	0	97	0.099	0	288	0.091	0.237	7,600	0.083	0.226	57,200	0	0.2	0

TABLE V.

Condensed Table of Experiments in Which the Heart and Pancreas Were Perfused Together Simultaneously.

Experiment No.	Weight of heart.	Dextrose after 10 min. perfusion.	1st hr.			2nd hr.			3rd hr.			4th hr.			Dextrose per gm. of heart recovered by hydrolysis.	Average error of duplicate determinations.	Dextrose disappeared from control perfusion.
			Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.			
	gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*	*per cent*	
30......	97	0.196	0.179	0.465		0.167	0.306	12,200	0.143	0.60	23,600	0.126	0.42	50,400	0.49	0.49	0
31......	128	0.399	0.333	1.28	50,000	0.286	0.92	200,000	0.273	0.212	500,000	0.272	0.042	800,000	0.27	0.5	0
32......	128	0.300	0.272	0.531	49,600	0.231	0.756	512,000	2½ hrs. 0.213	0.34					0.031	0.4	0
33......	102	0.100	0.077	0.557	960	0.058	0.45	16,700	0.041	0.395	108,460	0.025	0.384	356,000	0.19	0.32	0
37......	86	0.100	0.089	0.30	1	0.054	0.964	1	0.035	0.532	2	0.026	0.245	2	0.54	0.32	0
39......	98	0.200	0.165	0.872	1	0.142	0.57	0	0.128	0.356	4	0.123	0.113	26	0.47	0.34	0

TABLE VI.

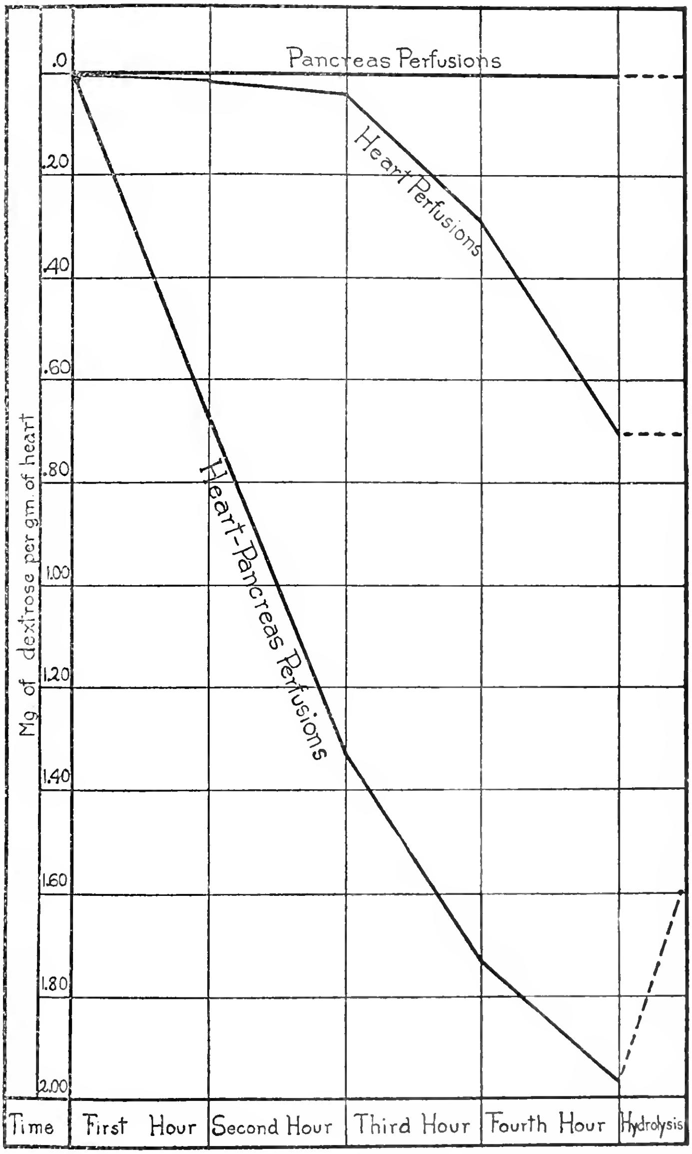
Table Combining the Results Given in Tables III, IV, and V.

	1st hr.	2nd hr	3rd hr.	4th hr.	Recovered by hydrolysis.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Average utilization of dextrose per gm. of pancreas in 3 perfusions.	0	0	0	0	0
Average utilization of dextrose per gm. of heart in 5 perfusions....	0.028	0.013	0.242	0.415	0
Average utilization of dextrose per gm. of heart in 6 heart-pancreas perfusions....................	0.667	0.661	0.406	0.200	0.332

2nd hour of perfusion, but from this point on, during the period of observation, it occurs in an increasing ratio. When the heart and pancreas are perfused together a striking loss of sugar occurs in the 1st hour, and towards the latter half of the experimental period of 4 hours this disappearance of sugar shows a decreasing ratio. Text-fig. 2 illustrates this point. The further fact also became apparent that hydrolysis of the final perfusates invariably gave a moderate but definite increase in the reducing substance. After deducting this amount of reducing substance recovered by hydrolysis, the amount of dextrose utilized by the heart in these experiments exceeded considerably that utilized by the heart alone. This is indicated in the tables and figures. In no instance did the control perfusion show any variation in reducing sugar beyond the limits of experimental error. From these experiments, then, it seemed that the pancreas not only enabled the heart in some direct way to utilize dextrose, but some alteration in the dextrose also occurred whereby it was changed in part at least to a non-reducing form which could be transformed back to dextrose again by hydrolysis.

Was This Utilization of Dextrose a Tissue Extract Phenomenon?

Was the disappearance of dextrose when the heart and pancreas were perfused together an effect similar to that obtained when muscle and pancreas extracts are allowed to act on dextrose? A number of heart-pancreas perfusions, which were considered as failures because



TEXT-FIG. 2. Curves showing the utilization of dextrose when the pancreas is perfused alone, when the heart is perfused alone, and when the two are perfused simultaneously. Note the increase in reducing substance in the heart-pancreas perfusions on hydrolysis.

TABLE VII.

The Effect of Pancreatic Perfusates and Pancreatic Extracts on Non-Beating Hearts.

Experiment No.	Procedure of experiment.	Weight of heart.	Dextrose after 10 min. perfusion.	1st hr.			2nd hr.			3rd hr.			4th hr.		
				Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.
		gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*	
36	Non-beating heart-pancreas perfusion......	80	0.100	0.100	0	4	0.099	0	0	0.099	0	0	0.100	0	0
43	Non-beating heart perfused with pancreas extract............	106	0.200										0.200	0	0
45	Non-beating heart perfused with pancreas extract............	94	0.146										0.145	0.002 per hr.	1,260
62	Heart and pancreas frozen then perfused.	165	0.199	0.200	0		0.200	0		0.200	0		0.200	0	

the heart stopped beating soon after the perfusion was begun, showed practically no utilization of dextrose. The results of four such experiments are given in Table VII. In Experiment 36 the heart stopped beating almost immediately at the beginning of the perfusion. In Experiment 62 the heart and pancreas were frozen on removal from the body and then perfused. In Experiments 43 and 45 the pancreas was removed and ground, with all aseptic precautions, extracted with Locke's solution, and filtered. The hearts were then perfused with this extract. The hearts stopped beating almost instantly and the perfusion pressures rose to over 250 mm. of mercury. The perfusions were continued above this pressure, however. In none of these four experiments was there any disappearance of dextrose over a 4 hour period.

Again, in all the heart-pancreas perfusions reported above, and others to be mentioned below, the final perfusates were placed in the thermostat with chloroform and toluene as preservatives. If the dextrose utilization had been due merely to the extracts of the heart and pancreas the disappearance of dextrose ought to continue in the fluid after perfusion. It was invariably found, however, that not only was there no subsequent disappearance of dextrose in these perfusates, but that they generally showed an increase in the amount of reducing substance present. The immediate cessation of glycolytic action was probably not due to the preservatives used, for the experiments of most of the workers from Cohnheim to Levene and Meyer have been based on the effect of tissue extracts preserved with similar antiseptics. It is also to be emphasized that if the disappearance of dextrose was due to the tissue extracts, some change might be expected in the control perfusions which had passed for 10 minutes through the vessels of the two organs. The control perfusions, however, did not show any change, and this fact is discussed further below. Finally, in the experiments of Levene and Meyer (14) this phenomenon occurred only with strengths of dextrose greatly in excess of the physiological amounts used in these experiments, and on hydrolysis they were able to obtain practically all the original sugar. In my experiments only a small portion of the reducing substance could be recovered by hydrolysis. It is believed, therefore, that the results of these experiments indicate that the utilization of dextrose in the

heart-pancreas perfusions in which the heart was actively beating was not a phenomenon similar to that obtained when muscle and pancreas extracts are allowed to act on more concentrated solutions of dextrose, but that in these experiments the utilization of dextrose was dependent on the activity of the living heart tissues.

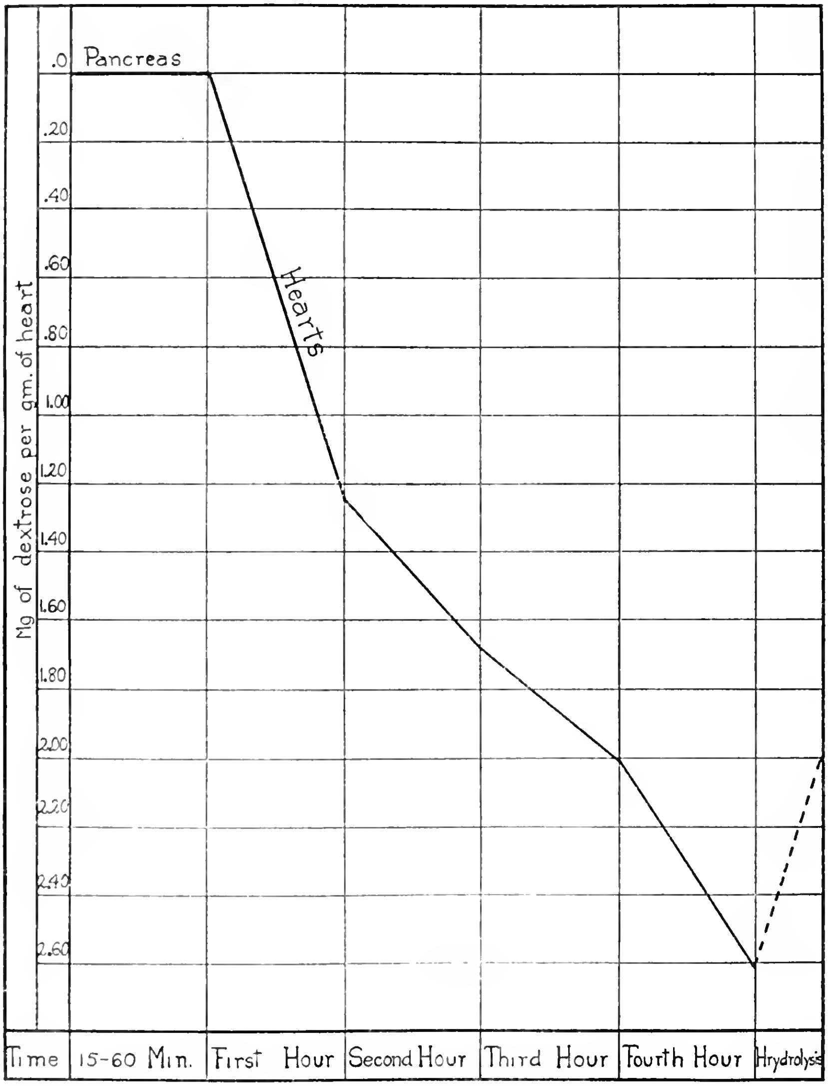
The Pancreas First Perfused and Subsequently the Heart Perfused with the Same Solution.

The question next arose as to whether the pancreas supplied anything to the perfusing Locke's solution which could subsequently cause the utilization of dextrose by the heart. Six experiments were conducted to determine this point. The results are given in Table VIII, and charted in Text-fig. 3. In Experiments 40 and 42 the pancreas was first perfused for 1 hour with Locke's solution containing dextrose. The dog was meanwhile kept alive under light ether anesthesia. The pancreas was then removed from the apparatus, and the heart substituted and perfused with the same solution that had been perfusing through the pancreas. The utilization of dextrose by the heart proceeded exactly as if the pancreas were in circuit, and hydrolysis of the final perfusate gave an increase in the reducing substance. Four experiments were then carried out in which the pancreas was first perfused for a period of 15 to 40 minutes with a Locke's solution containing no dextrose. The pancreas was then removed from the apparatus and sufficient dextrose added to the perfusing solution to give approximately the desired per cent. After allowing it to mix thoroughly in the apparatus, perfusion of the heart was begun. Again all the experiments showed rapid dextrose utilization and, as in the heart-pancreas perfusions, hydrolysis of the final perfusates increased their reducing properties. Two facts thus seemed evident. First, the pancreas did not alter the reducing properties of the dextrose passing through its vessels. Second, the pancreatic factor causing the rapid disappearance of sugar was contained in the perfusate and continued to act after removal of the pancreas. The pancreas, therefore, had furnished some constituent to the perfusing liquid which greatly accelerated the utilization of sugar by the heart muscle.

TABLE VIII.

Experiments in Which the Heart Was Perfused with a Solution Which Had Previously Been Perfused through the Pancreas. In the Last Four Experiments the Pancreas Was Perfused with Locke's Solution Containing No Dextrose. After Removal of the Pancreas Sufficient Dextrose Was Added to the Perfusate to Give the Desired Per Cent.

Experiment No.	Length of time pancreas perfused.	Dextrose utilized by pancreas.	Weight of heart.	Dextrose after 10 min. heart perfusion.	1st hr.			2nd hr.			3rd hr.			4th hr.			Dextrose per gm. of heart recovered by hydrolysis.
					Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	Dextrose.	Dextrose disappeared per gm. of heart.	Bacteria per cc.	
			gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*
40	1 hr.	0	82	0.100	0.091	0.277	12	0.077	0.41	220	0.064	0.38	164	0.048	0.600	64	0.422
42	1 "	0	81	0.199	0.167	0.99	5	0.153	0.442	36	0.153	0	121	0.123	0.87	3	0.5
44	40 min.	0	119	0.297	0.160	2.76	0	0.133	0.555	0	0.109	0.40	1	0.092	0.28	5	0.43
46	15 "	0	91	0.246	0.176	2.1	0	0.170	0.158	0	0.121	1.22	0	0.109	0.28	0	0.94
47	40 "	0	105	0.096	0.072	0.63	0	0.059	0.386	0	0.051	0.17	0	0.051	0	0	0.465
48	40 "	0	105	0.30	0.272	0.761	0	0.24	0.605	0	0.217	0.422	0	0.171	0.86	0	0.55



TEXT-FIG. 3. The average curve of the six experiments in Table VIII. The pancreas was first perfused for periods varying from 15 to 60 minutes, and then removed from the apparatus and the heart of the same dog substituted. There was no dextrose utilization by the pancreas, but when the heart was subsequently perfused with the fluid which had been perfused through the pancreas, the heart-pancreas type of dextrose utilization occurred.

Effect of Heat on Pancreatic Perfusates.

If this pancreatic factor accelerating dextrose utilization by the heart is an enzyme, it should be affected by heat; while if it has a more stable character like adrenalin it should not be affected by heat to any extent. The five experiments on this point are tabulated in Table IX and shown in Text-fig. 4. In all the experiments the pancreas was perfused for 30 minutes and the perfusate was then removed from the apparatus. After completion of the pancreas perfusion the apparatus was carefully washed and sterilized in the steam sterilizer for 30 minutes. The heart was then perfused with the pancreatic perfusate which meanwhile had been subjected to various degrees of heating. In Experiments 51 and 52 the pancreatic perfusate was just brought to the boiling point and cooled, while in Experiment 55 the perfusate was boiled for 10 minutes. In Experiments 53 and 54 the pancreatic perfusates were heated to 56°C. for 30 minutes on a water bath, then cooled and perfused through the heart. In all these experiments the heart continued to beat during the 4 hour period. The results were definite, as shown in Text-fig. 4. When the pancreatic perfusate was heated to boiling it lost its power of enabling the heart to utilize dextrose. The utilization of dextrose proceeded practically as if the heart were being perfused alone. Heating the pancreatic perfusate to 56°C. for 30 minutes, however, did not cause any inactivation. Subsequent perfusion through the heart gave the typical heart-pancreas type of dextrose utilization. These experiments, therefore, indicated that the pancreatic factor has the characteristic of an enzyme in being inactivated by boiling temperature.

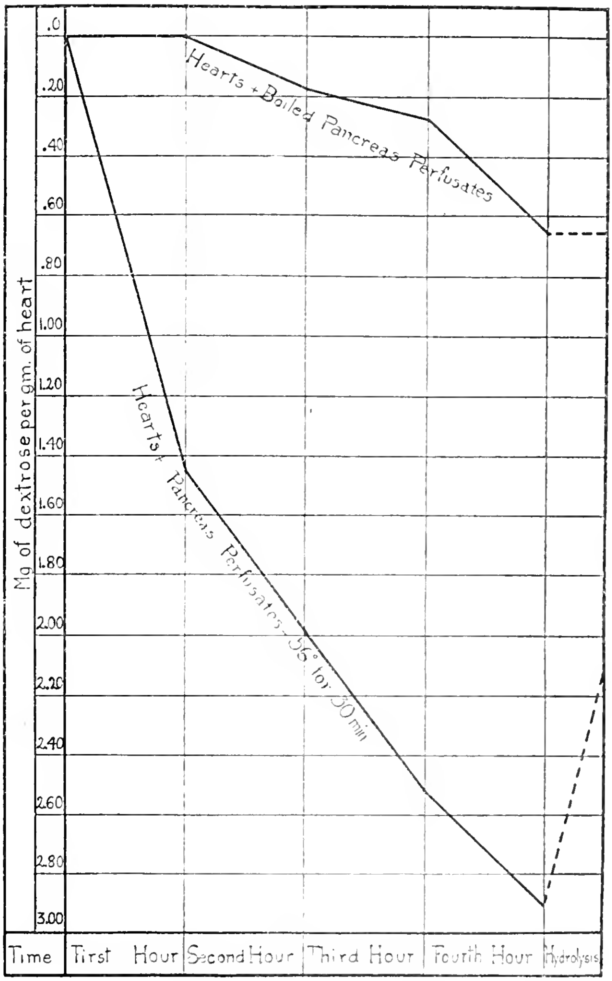
Amount of Pancreatic Perfusate Necessary to Enable the Heart to Utilize Dextrose.

In the experiments shown in Table VIII in which the pancreas was first perfused and the same solution was subsequently perfused through the heart, the entire pancreatic perfusate of 250 cc. was used. The question arose as to the minimum amount of pancreatic perfusate necessary to enable the heart to utilize dextrose immediately. Four experiments were carried out as shown in Table X and

TABLE IX.

Condensed Table of Experiments Showing the Effect of Heat on the Pancreatic Perfusate.

Experiment No.	Pancreas perfused 30 min. Perfusate removed and apparatus sterilized. Heart then perfused with pancreatic perfusate treated as follows:	Dextrose utilized by pancreas.	Weight of heart.	Dextrose after 10 min. heart perfusion.	1st hr. Dextrose.	1st hr. Dextrose disappeared per gm. of heart.	1st hr. Bacteria per cc.	2nd hr. Dextrose.	2nd hr. Dextrose disappeared per gm. of heart.	2nd hr. Bacteria per cc.	3rd hr. Dextrose.	3rd hr. Dextrose disappeared per gm. of heart.	3rd hr. Bacteria per cc.	4th hr. Dextrose.	4th hr. Dextrose disappeared per gm. of heart.	4th hr. Bacteria per cc.	Dextrose per gm. of heart recovered by hydrolysis.
			gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*
51	Pancreas perfusate brought to boiling temperature........................	0	50	0.119	0.119	0	0	0.108	0.523	0	0.093	0.139	9	0.085	0.204	4	0.03
52	Pancreas perfusate brought to boiling temperature........................	0	63	0.120	0.120	0	0	0.120	0	0	0.120	0	0	0.099	0.711	9	0
55	Pancreas perfusate boiled for 10 min...	0	62	0.200	0.199	0	5	0.200	0	6	0.196	0.14		0.188	0.274	31	0
53	Pancreas perfusate heated to 56° C. for 30 min...........................	0	72	0.200	0.154	1.55	1	0.137	0.563	0	0.126	0.337	4	0.113	0.496	17	0.71
54	Pancreas perfusate heated to 56° C. for 30 min...........................	0	60	0.200	0.166	1.35	1	0.153	0.496	2	0.133	0.733	5	0.125	0.286	3	0.86

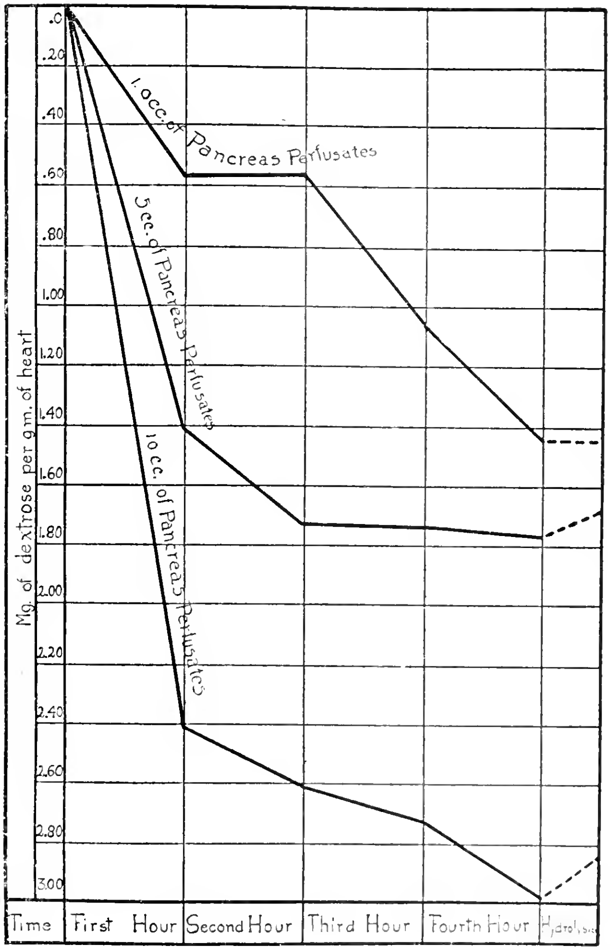


TEXT-FIG. 4. Chart of Table IX showing the effect of heat on the pancreatic perfusates. The upper curve shows the utilization of dextrose in the experiments in which the pancreatic perfusate was heated to boiling and cooled before perfusion through the heart, while the lower curve shows what occurred when the pancreatic perfusate was only heated to 56°C. for 30 minutes.

TABLE X.

Results of Experiments to Determine the Amount of Pancreatic Perfusate Necessary to Enable the Heart to Utilize Dextrose Immediately.

Experiment No.	Pancreas perfused 30 min. Perfusate removed and apparatus sterilized. Heart then perfused with the following mixtures.	Dextrose utilized by pancreas.	Weight of heart.	Dextrose after 10 min. heart perfusion.	1st hr. Dextrose.	1st hr. Dextrose disappeared per gm. of heart.	1st hr. Bacteria per cc.	2nd hr. Dextrose.	2nd hr. Dextrose disappeared per gm. of heart.	2nd hr. Bacteria per cc.	3rd hr. Dextrose.	3rd hr. Dextrose disappeared per gm. of heart.	3rd hr. Bacteria per cc.	4th hr. Dextrose.	4th hr. Dextrose disappeared per gm. of heart.	4th hr. Bacteria per cc.	Dextrose per gm. of heart recovered by hydrolysis.
			gm.	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*
49	Pancreatic perfusate 10 cc. Locke's solution 240 "	0	70	0.265	0.176	3.07	0	0.173	0.122	0	0.165	0.244	0	0.149	0.49	0	0
50	Pancreatic perfusate 10 " Locke's solution 240 "	0	119	0.284	0.175	1.76	0	0.157	0.27	0	0.158	0	0	0.158	0	0	0.248
57	Pancreatic perfusate 5 " Locke's solution 245 "	0	83	0.200	0.153	1.41	27	0.143	0.316	5	0.142	0.011	25	0.141	0.032	128	0.083
59	Pancreatic perfusate 1 " Locke's solution 249 "	0	75	0.200	0.182	0.564	0	0.183	0	0	0.166	0.50		0.154	0.37	89	0



TEXT-FIG. 5. Chart of Table X showing the result of adding different amounts of the pancreatic perfusate to a perfusion of the heart from the same dog.

Text-fig. 5. The pancreas was first perfused with 250 cc. of Locke's solution containing 0.2 per cent of dextrose. This perfusate was then removed and the apparatus was washed and sterilized for 30 minutes to inactivate any adherent pancreatic substance. The heart of the same animal was then perfused with a fresh solution to which varying amounts of the pancreatic perfusate had been added, the total volume of the perfusate being made up to 250 cc. As seen in the curves of Text-fig. 5, when 10 cc. of the pancreatic perfusate were added, the curve of dextrose utilization by the heart is practically the same as if the entire 250 cc. had been used. Hydrolysis of the final perfusates, however, did not give as definite an increase in reducing substance as in the typical heart-pancreas perfusions. When 5 cc. and 1 cc. were used, though the utilization of dextrose began within the 1st hour, it did not proceed as rapidly as when the larger amounts were used. These experiments indicate that even 1 cc. of a 250 cc. pancreatic perfusate added to 249 cc. of Locke's solution has a distinct effect in enabling the heart to utilize dextrose immediately.

Instability of the Pancreatic Perfusate after Perfusion.

It was hoped to carry out a number of other experiments which would depend on the ability of the pancreatic perfusate to maintain its activity when kept for 24 hours or more without a preservative. To test this, three experiments were carried out. The figures are shown in Table XI. A pancreas was perfused with 250 cc. of Locke's solution for 30 minutes and the perfusate was placed on ice for 48 hours in one experiment and 24 hours in two experiments. At the end of that time the heart of another dog was perfused with the entire pancreatic perfusate, but with the result that only the heart type of dextrose utilization occurred. It hardly seemed probable that this could be due to the fact that different dogs were used, but rather that the enzyme-like substance had lost its activity during these short periods of time. That the perfusates used were originally fully active was demonstrated by the fact that they were the perfusates which had previously been shown to be highly active.

TABLE XI.

Experiments to Determine whether the Pancreatic Perfusate when Allowed to Stand in the Ice Box Loses Its Power to Enable the Heart to Utilize Dextrose Immediately.

Experiment No.	Heart perfused with pancreatic perfusate from experiment No.	Pancreatic perfusate in ice box.	Weight of heart.	Dextrose after 10 min. heart perfusion.	1st hr. Dextrose.	1st hr. Dextrose disappeared per gm. of heart.	1st hr. Bacteria per cc.	2nd hr. Dextrose.	2nd hr. Dextrose disappeared per gm. of heart.	2nd hr. Bacteria per cc.	3rd hr. Dextrose.	3rd hr. Dextrose disappeared per gm. of heart.	3rd hr. Bacteria per cc.	4th hr. Dextrose.	4th hr. Dextrose disappeared per gm. of heart.	4th hr. Bacteria per cc.	Dextrose per gm. of heart recovered by hydrolysis.
		hrs.	*gm.*	*per cent*	*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*per cent*	*mg.*		*mg.*
56	54	48	59	0.200	0.200	0	4	0.184	0.27	28	0.166	0.26	344	0.151	0.18	824	0
58	57	24	56	0.200	0.200	0	980	0.200	0	220	0.167	1.33	312	0.148	0.777	920	0
60	59	24	54	0.195	0.191	0.18	54	0.183	0.32	12	0.154	1.22	9	0.138	0.69	44	0

The Latency of Sugar Utilization.

An interesting finding which should be mentioned is the fact that in the heart-pancreas perfusions no dextrose utilization ever seemed to have occurred in the first 10 minutes of perfusion, the period which was allowed to elapse before filling the control apparatus. If the dextrose had been used by the heart in this time at the same rate that it was used in the following hour, the utilization should at least have been evident. Even in the experiments where the pancreas was first perfused and the heart subsequently perfused with the same fluid, there was no evident utilization of sugar during the first 10 minutes. In such experiments the hearts must have been supplied immediately by an abundance of the pancreatic enzyme. From my experiments it cannot be stated how much more than 10 minutes elapsed before utilization of dextrose began, for an hour was allowed to pass before making another reading. It would seem, therefore, that there is a certain latent period before the heart begins to use dextrose, even though the dextrose is accompanied by an abundance of the pancreatic enzyme.

SUMMARY.

The experiments indicate that the pancreas, when perfused aseptically with Locke's solution containing physiological concentrations of dextrose, does not alter the reducing properties of the perfused solution. The pancreas, however, seems to supply something to the Locke's solution circulating through its arteries which in some way brings about a utilization of sugar by the living heart to an extent that does not occur with the heart alone. This pancreatic substance possesses some of the characteristics of an enzyme. It is inactivated by boiling; it is unstable, rapidly becoming inactive on standing; it acts in small amounts; it causes a great acceleration in the rate of a reaction which otherwise proceeds slowly, and the rate of reaction diminishes as the reaction proceeds. Thus this substance has more of the characteristics of an enzyme than of a stable internal secretion like that of the adrenal glands. The disappearance of sugar was dependent upon the presence of living heart tissue, and it ceased as soon as the perfusate was removed from the heart-pancreas circula-

tion and did not occur at all when a pancreatic perfusate was passed through a non-beating heart. This result indicates that the reaction is not similar to that obtained when muscle and pancreas extracts act on more concentrated solutions of dextrose.

The living heart in the presence of the pancreatic factor and dextrose, is responsible for two effects. First, a condensation of the sugar to a non-reducing form that yields again a simple sugar on hydrolysis or by simply standing, with a preservative, at 37°C. for 24 hours. Second, a disappearance of sugar which is probably due to its destruction by hydrolysis or oxidation. After deducting the reducing sugar in the heart-pancreas perfusions which could be recovered by hydrolysis, the amount of sugar which had actually disappeared exceeded that which was used by the heart when perfused with dextrose alone. As to the fate of this portion of the sugar, no definite evidence was obtained.

The question arises as to whether this substance obtained from the perfused pancreas is identical with the hypothetical internal secretion of the pancreas so essential in sugar metabolism. That there is an internal secretion of the pancreas which can be obtained by this method, and that in some way it accelerates the utilization of sugar by the living heart, seems evident. Though the conclusions are based on the heart and pancreas isolated from the numerous interrelating factors occurring in the body, the evidence suggests, at least, that the substance or substances obtained by perfusing the pancreas may be concerned in the normal activity of the pancreas upon sugar metabolism.

BIBLIOGRAPHY.

1. Hédon, E., *Compt. rend. Soc. biol.*, 1909, lxvii, 792.
2. Carlson, A. J., and Ginsburg, H., *Am. J. Physiol.*, 1915, xxxvi, 280.
3. Forschbach, J., *Deutsch. med. Woch.*, 1908, xxxiv, 910.
4. Carlson, A. J., and Drennan, F. M., *Am. J. Physiol.*, 1911, xxviii, 391.
5. Verzár, F., and von Fejér, A., *Biochem. Z.*, 1913, liii, 140.
6. Woodyatt, R. T., quoted by Macleod, J. J. R., Harvey Lecture 1914, "Physiological Pathology of Glycosuria."
7. Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.
8. Scott, E. L., *Am. J. Physiol.*, 1912, xxix, 306.
9. Cohnheim, O., *Z. physiol. Chem.*, 1903, xxxix, 336.

10. Claus, R., and Embden, G., *Beitr. chem. Phys. u. Path.*, 1905, vi, 214, 343.
11. Arnheim, J., and Rosenbaum, A., *Z. physiol. Chem.*, 1903–04, xl, 220.
12. Dewitt, L. M., *J. Exp. Med.*, 1906, viii, 193.
13. Hall, G. W., *Am. J. Physiol.*, 1907, xviii, 283.
14. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1911, ix, 97.
15. Hustin, A., *Arch. internat. physiol.*, 1912, xii, 318.
16. de Meyer, J., *Arch. internat. physiol.*, 1910, ix, 1.
17. Müller, J., *Z. allg. Physiol.*, 1904, iii, 282.
18. Locke, F. S., and Rosenheim, O., *J. Physiol.*, 1907, xxxvi, 205.
19. Camis, M., *Z. allg. Physiol.*, 1908, viii, 371.
20. Rohde, E., *Z. physiol. Chem.*, 1910, lxviii, 181.
21. Gayda, T., *Z. allg. Physiol.*, 1912, xiii, 1.
22. Knowlton, F. P., and Starling, E. H., *J. Physiol.*, 1912, xlv, 146.
23. Neukirch, P., and Rona, P., *Arch. ges. Physiol.*, 1912, cxlviii, 285.
24. Maclean, H., and Smedley, I., *J. Physiol.*, 1912, xlv, 470.
25. Maclean and Smedley, *J. Physiol.*, 1912, xlv, 462.
26. Mansfeld, G., *Zentr. Physiol.*, 1913, xxvii, 267.
27. Patterson, S. W., and Starling, E. H., *J. Physiol.*, 1914, xlvii, 137.
28. Cruickshank, E. W. H., *J. Physiol.*, 1913, xlvii, 1.
29. Cruickshank, E. W. H., and Patterson, S. W., *J. Physiol.*, 1913–14, xlvii, 381.
30. Starling, E. H., and Evans, C. L., *J. Physiol.*, 1914–15, xlix, 67.
31. Underhill, F. P., and Prince, A. L., *J. Biol. Chem.*, 1914, xvii, 299.
32. Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

A CONTRIBUTION TO THE EPIDEMIOLOGY OF LOBAR PNEUMONIA.

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Statistics demonstrate that of all the acute infectious diseases in the United States, lobar pneumonia is responsible for the greatest number of deaths. The incidence of this disease in the larger cities during the winter months is extraordinarily high, and health reports indicate that during the past three decades there has been but little evidence of a tendency to a spontaneous decrease in its morbidity. Scientific investigation of pneumonia has dealt largely with methods of therapy, and but little or no effort has been expended in the elaboration of means of prevention. In recent years the study of the bacteriology of lobar pneumonia has developed new facts, which make it advisable to study the epidemiology of the disease, in the hope that such an investigation may bring to light important factors concerned in its transmission from one individual to another.

Dochez and Avery[1] have reported observations which suggest that in a majority of instances of lobar pneumonia, the infection is acquired by transmission of the infectious agent from a recovered case which still harbors in the mouth secretions the organism responsible for the disease, or from a healthy carrier of pathogenic pneumococci. The validity of this assumption depends upon the following factors. Lobar pneumonia in 65 per cent of cases is due to specific types of pneumococci possessed of high pathogenicity, which do not occur in the buccal secretions of normal individuals, except in instances where there has been intimate association with persons suffering from the disease. Although pneumococci are present in the mouths of 60 per cent of normal individuals, the organisms present are readily

[1] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxii, 105.

distinguishable from the highly parasitic types of pneumococcus which cause the severe forms of lobar pneumonia. The organisms found in normal mouths, moreover, occasion but a relatively small percentage of the total cases of this disease.

In the present paper are reported the facts obtained during an investigation, which included, first, a study of the varieties of pneumococci concerned in the production of cases of lobar pneumonia admitted to the Hospital of The Rockefeller Institute during the past 4 years; second, a study of the varieties of pneumococci in the mouths of normal individuals; third, the frequency of occurrence of disease-producing types of pneumococci in the mouths of normal individuals; fourth, the frequency of the occurrence of disease-producing types of pneumococci in the mouth secretions of healthy persons associated with cases of lobar pneumonia; and fifth, a study to determine the period of time during which convalescents harbor in the mouth the types of pneumococci responsible for the disease.

Varieties of Pneumococci Concerned in the Production of Lobar Pneumonia.

During the past 4 years a careful study has been carried on of the types of pneumococci isolated from cases of lobar pneumonia admitted to the wards of the Hospital of The Rockefeller Institute. These organisms have been isolated from the sputum, blood, or lung directly, and in many instances from more than one of these sources, and classified according to the biologic groups previously described.[2] The results of this study are shown in the following table.

TABLE I.

Types of Pneumococci Causing Lobar Pneumonia.

Types.	1912-13	1913-14	1914-15	1915-16	Total.	Per cent.
I	29	22	21	33	105	33.54
II	8	28	35	28	99	31.62
III	7	6	9	13	35	11.18
IV	16	13	17	28	74	23.64

[2] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727. Dochez and Avery, *J. Exp. Med.*, 1915, xxi, 114.

During the year 1915–16 the incidence of Types II a, II b, II x, subvarieties of Group II described by Avery,[3] has also been studied. In 102 cases of lobar pneumonia Type II a occurred five times, Type II b not at all, and Type II x once.

Examination of Table I reveals the constancy with which the various types of pneumococci occur from year to year. In the years 1912–13 and 1915–16 Type I was the dominant one. In 1913–14 and 1914–15 the percentage incidence of Type II increased, making this group the dominant type during these years. The percentage incidence of cases due to Types III and IV has remained fairly constant from year to year. Inasmuch as it has been previously demonstrated that organisms of the various types differ in their virulence for human beings, changes in mortality from year to year may be dependent upon the dominance of a given type. Of the 313 cases of lobar pneumonia presented in Table I, 204, or 65 per cent, were caused by pneumococci belonging to Types I and II. Since these types are not found in the normal mouth except in the case of carriers, they are regarded as strictly disease-producing organisms, and infection with organisms of either type probably occurs only as a result of direct or indirect contact with a case of pneumonia infected with an organism of one of these types. Recent studies show that pneumococci of Type III are fairly common in the mouth flora of healthy individuals and infections with organisms of this type may be autogenic in nature. The atypical organisms belonging to Type II which induce lobar pneumonia are indistinguishable from the similar organisms which may be the dominant type of pneumococcus found in the normal mouth, and infection with these organisms may also be autogenic.

Varieties of Pneumococcus in the Normal Mouth.

The pneumococcus occurs in the mouth secretions of normal individuals in a large percentage of cases. In some individuals the organism may be present constantly in the mouth secretions, while in others it may be present at one time and absent at another. In

[3] Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

Table II is presented a study of types of pneumococcus recovered from the saliva of 398 healthy persons.

TABLE II.

Types of Pneumococcus Isolated from the Sputum of Normal Individuals.

Pneumococcus.	Incidence.	Per cent.
Type I	4	2.16
" II	4	2.16
" II a	0	
" II b	11	5.84
" II x	15	7.79
" III	44	23.40
" IV	110	58.51
Pneumococcus present.	172	
" absent.	226	
	398	

Among 398 normal individuals examined pneumococcus was obtained in 172 instances. In 226 individuals no pneumococcus could be demonstrated in the mouth at the time of examination. In four instances the pneumococcus isolated was Type I. In three of these individuals a history of close association with a case of lobar pneumonia was obtained, whereas in one no such association could be traced. From four individuals, organisms of Type II were obtained. Three of these individuals gave a history of intimate contact with cases of lobar pneumonia of the same type, and in one no such association could be established. Atypical Type II organisms were isolated twenty-six times. Pneumococci of Type II b were found eleven times and of Type II x fifteen times. In view of the relative frequency with which these atypical Type II organisms can be obtained from the sputum of normal persons who give no history of association with individuals suffering from lobar pneumonia due to similar types, these organisms, like those of Group IV, must be considered as readily adapting themselves to existence in the normal mouth secretions.

Of the 398 normal individuals examined, in no instance was a pneumococcus of Subgroup II a isolated, while this organism was found in five cases of pneumonia.

At the time of publication of previous papers dealing with the epidemiology of lobar pneumonia, biologic studies dealing with the organism of Type III had not been carried sufficiently far to determine whether organisms of this type isolated from normal mouths differed in their immunological reactions from those of the same type obtained from cases of lobar pneumonia. In the present study of 398 normal individuals, the pneumococcus of Type III (*Pneumococcus mucosus*) was present in the sputum in 44 instances. Through the courtesy of the New York State Board of Health we have recently obtained an immune serum which both agglutinates and protects animals against *Pneumococcus mucosus*. With this serum a study of the immunological properties of all the cases of this type of organism which have been isolated from normal mouths, as well as of those which have been isolated from cases of pneumonia, has been made and no biologic differences have been detected. A serum prepared by the immunization of a horse to *Pneumococcus mucosus* isolated from a case of lobar pneumonia agglutinates and protects animals against infection from all strains of this type isolated from patients with pneumonia as well as almost all those isolated from normal persons. Curiously enough, therefore, this organism, which is to be considered as one of the fixed biologic types and which possesses for human beings the greatest virulence of all pneumococci, seems to be able to accommodate itself to saprophytic existence in the normal mouth for long periods of time without producing disease. Although virulent organisms of this type occur in normal sputum with considerable frequency, they are responsible for a relatively small number of cases of lobar pneumonia. Table III shows how long certain normal individuals may harbor *Pneumococcus mucosus* in their mouths without suffering from any recognizable infection.

Table III shows that *Pneumococcus mucosus* may persist in the mouth secretions of normal individuals over long periods of time. The longest interval noted in the present study was over 3 months, and evidence indicates that in some instances this organism may be harbored in the sputum for a year or more with impunity.

Previous studies indicate that the commonest type of pneumococcus found in the normal mouth is Type IV, and in this study of 398 healthy persons organisms of this type were found 110 times.

TABLE III.

Persistence of Pneumococcus mucosus (Type III) in Normal Mouths.

Case No.	Interval.	Type of pneumococcus.
1	1st examination.	III
	4 days.	III
	8 "	III
	22 "	III
	28 "	III
	35 "	III
	42 "	III
	49 "	III
	56 "	No pneumococcus.
	66 "	III
	71 "	No pneumococcus.
	78 "	III
	98 "	III
2	1st examination.	III
	7 days.	No pneumococcus.
	14 "	III
	21 "	III
	49 "	III
	56 "	No pneumococcus.
3	1st examination.	III
	7 days.	III
	14 "	III
	21 "	III
	49 "	III
	77 "	III
	91 "	III
4	1st examination.	III
	15 days.	III
	22 "	III
	29 "	No pneumococcus.
	36 "	III

The technique of isolating the pneumococcus from the mouth secretions of healthy individuals has been that commonly employed as a routine procedure in the isolation of pneumococci from the sputum of pneumonia patients.

The saliva of the individuals studied was injected into the peritoneal cavity of white mice, and the pneumococcus was recovered at autopsy from the peritoneal exudate or heart's blood. In order to determine whether the pneumococcus could be cultivated as readily

from the direct sputum culture on blood agar, and whether the pneumococcus thus recovered differed from the organism isolated by animal passage, film preparations of 116 specimens of sputum were made on the surface of blood agar plates, as well as injected intraperitoneally into mice. In 58 specimens pneumococci were neither obtained from the mouse nor from the direct culture. In 47 instances pneumococci were recovered by mouse passage, whereas none was obtained by direct culture. In only 11 instances were pneumococci isolated both by animal inoculation and direct sputum culture, and in each of these cases the type obtained by mouse passage corresponded with that obtained by direct culture.

Incidence of Carrier Condition in Healthy Individuals in Contact with Patients Suffering from Lobar Pneumonia.

Dochez and Avery[4] have shown that a considerable percentage of individuals coming intimately in contact with active cases of lobar pneumonia due to infection with pneumococci of Type I or Type II, carry in their mouth secretions organisms of the same type as that causing the infection. Table IV presents a study of the types of pneumococci found in the mouth secretions of persons in intimate association with certain of the cases of lobar pneumonia admitted to the Hospital of The Rockefeller Institute during the years 1915–16. These include all the cases in which contacts were examined from this point of view.

Table IV shows the incidence and type of pneumococci found among the associates of the cases of lobar pneumonia. In 24 of these cases infection was due to pneumococci of Type I, and in 17 to pneumococci of Type II. In the normal mouth secretions of 44 contact cases infected with Type I pneumococci, pneumococci of a similar type were found in two instances, 8.33 per cent of such cases showing at least one contact among the associates. In all, 44 individuals in contact with pneumonia cases infected with Type I pneumococci were studied, and 4.5 per cent of these persons were found to harbor in their saliva pneumococci of a similar type. Among the associates of the 17 cases infected with Type II pneumococci, three

[4] Dochez and Avery, *J. Exp. Med.*, 1915, xxi, 114.

TABLE IV.

Incidence of Carrier Condition in Healthy Individuals in Contact with Lobar Pneumonia.

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Day of disease.	Type found in associates.
2,443	II	Sister.	2	II and IV
		"	2	II b
2,448	II	Wife.	3	No pneumococcus.
2,456	I	Sister.	29	IV
2,460	I	"	5	III
		"	5	No pneumococcus.
		Brother.	5	II b
2,477	II	"	6	IV
		"	26	IV
		"	22	III
		Sister.	6	III
		"	13	II x and IV
2,493	II	Brother.	4	II
		"	3	IV
		"	3	III
		"	28	No pneumococcus.
		Sister.	4	" "
		"	4	" "
2,497	I	Wife.	2	IV
		Brother.	5	IV
2,519	II	"	8	No pneumococcus.
		"	14	" "
2,521	II	Wife.	7	II
2,524	II	Sister.	9	No pneumococcus.
		"	11	IV
2,527	I	Husband.	8	No pneumococcus.
		"	9	IV
2,537	II	Mother.	5	IV
		"	6	IV
		"	13	No pneumococcus.
		"	18	" "
		Father.	4	" "
		"	18	II x
2,551	I	Wife.	5	No pneumococcus.
		"	10	" "
2,553	I	Mother.	4	" "
		"	9	" "
		"	16	" "
		Sister.	9	II b
		"	16	No pneumococcus.
2,556	I	Wife.	8	" "

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Day of disease	Type found in associates.
2,557	I	Mother.	4	No pneumococcus.
		Sister.	9	" "
2,558	I	Wife.	8	IV
		"	13	IV
		"	21	IV
2,560	I	Mother.	11	No pneumococcus.
2,571	I	Brother.	3	IV
		Sister.	3	IV
		"	16	I
2,572	II	Daughter.	9	IV
		Son.	10	IV
2,573	II	Brother.	2	IV
		Father.	20	No pneumococcus.
		Mother.	21	" "
2,576	I	Wife.	3	IV
		"	17	No pneumococcus.
		Sister.	24	III and IV
		"	24	No pneumococcus.
2,581	II	Wife.	10	IV
		Sister.	16	IV
2,586	II	Mother.	3	IV
		Father.	3	No pneumococcus.
		"	15	IV
		Brother.	8	III
		Sister.	8	IV
		"	15	IV
		"	22	No pneumococcus.
2,587	I	Husband.	6	II x
2,589	I	Wife.	12	IV
		"	19	IV
2,595	I	Sister.	6	No pneumococcus.
		Wife.	6	II
		"	9	II
		"	17	II
		"	23	II
		"	25	II
		"	31	II
		"	33	II
		"	34	II and III
		"	38	II
		"	39	II
2,597	I	Sister.	11	No pneumococcus.
		Brother.	18	" "

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Day of disease.	Type found in associates.
2,602	I	Cousin.	6	No pneumococcus.
		Mother.	5	IV
		"	11	IV
2,604	II a	Sister.	18	III
		Brother.	11	II x
2,608	I	Wife.	5	III
		"	16	III
		"	24	III
		"	52	No pneumococcus.
		"	65	IV
		Brother-in-law.	52	No pneumococcus.
		Sister.	59	III and IV
2,611	II	"	1	II x
		"	22	II x
		Wife.	2	II b and III
		"	16	III
		"	36	III and IV
		Daughter.	1	IV
		Niece.	22	No pneumococcus.
2,615	II	Sister.	2	IV
		"	13	No pneumococcus.
		Brother.	7	" "
		"	13	IV
		Wife.	2	No pneumococcus.
		"	21	III
		"	27	No pneumococcus.
2,622	II	Daughter.	12	" "
		Husband.	14	" "
		Daughter.	85	" "
2,623	I	Wife.	5	" "
		"	9	II b
		"	17	II b
		"	23	II b
		"	30	IV
		"	38	II b
		Nephew.	23	III
		Niece.	23	No pneumococcus.
		Friend.	23	" "
2,625	I	Sister.	15	IV
2,626	II	"	10	No pneumococcus.
		Father.	26	II b

TABLE IV—*Concluded.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Day of disease.	Type found in associates.
2,630	I	Father.	2	No pneumococcus.
		Mother.	9	" "
		"	16	" "
		"	24	" "
		"	32	" "
2,632	I	"	25	" "
		Father.	25	IV
		Sister.	37	IV
2,647	I	Wife.	2	No pneumococcus.
		Sister.	6	IV
		"	20	IV
2,652	I	Mother.	10	I

Summary.

Type.	No. of pneumonia cases studied.	No. of cases giving rise to carriers.	Per cent.	Total contacts examined.	No. of positive contacts.	Per cent.
I..............	24	2	8.33	44	2	4.5
II..............	17	3	17.64	40	3	7.5
Total..........	41	5	12.19	84	5	5.95

(17.64 per cent) were found to harbor in their mouths pneumococci of Type II. Altogether 40 contacts of cases due to Type II pneumococci were examined and from the sputum of 7.5 per cent of these, pneumococci of Type II were isolated. From a total of 41 cases of lobar pneumonia due to Type I and Type II, 5 gave rise to the carrier condition in at least one of their immediate associates, 12.19 per cent. The total number of contacts examined was 84, and of these 5, or 5.95 per cent, showed either pneumococci of Type I or Type II in their sputum. In one instance pneumococci of Type II were isolated from the sputum of the wife of a patient who suffered from pneumonia due to pneumococci of Type I. This is the only instance in our experience in which a pneumococcus of Type I or Type II has been isolated from the mouth of a contact where the type of organism found in the contact has differed from that isolated

from the patient. It is of interest in connection with this example that during convalescence the Type I pneumococcus disappeared from the sputum of the patient, and a pneumococcus of Type II then appeared in his mouth flora. A possible explanation is that the wife was a chronic carrier of Type II pneumococcus and that during convalescence, during which period the patient was in close contact with his wife, he secondarily acquired this type from her.

In this table no study of the carrier condition in associates of patients of Type III is recorded. Dochez and Avery in their report assumed the Type III pneumococcus to be a strictly disease-producing organism and not an inhabitant of the normal mouth secretions. Subsequent studies have shown that pneumococci of Type III are found so frequently in the mouths of normal individuals who have not associated with cases of lobar pneumonia that pneumococci of this type must be regarded as normal inhabitants of the healthy mouth. In view of these findings it would seem that the occurrence of this type of pneumococci in the mouths of individuals associated with cases of lobar pneumonia due to other types is to be expected and cannot be regarded as evidence against the epidemiological significance of the occurrence of carriers of pneumococci of Types I and II.

In this table are shown the results of repeated observations of the types of pneumococci in the mouths of a number of normal individuals. These observations bring to light the facts that the sputum type of pneumococcus may be present at one time and absent at another, that the types of pneumococci present may change from time to time, and also that more than one variety of sputum pneumococcus may be present at the same time.

Persistence during Convalescence of the Type of Pneumococcus Causing the Disease.

Previous study has demonstrated the fact that during the period of recovery from lobar pneumonia the type of pneumococcus causing the disease disappears from the mouth flora and in many instances is subsequently replaced by a pneumococcus of the variety commonly found in normal sputum. These facts are further corroborated by the evidence presented in Table V.

TABLE V.

Persistence during Convalescence of the Type of Pneumococcus Causing the Disease.

Case No.	Type of pneumococcus during height of disease.	Day of disease.	Type of pneumococcus recovered.
2,456	I	14	No pneumococcus.
		27	" "
2,464	I	31	" "
2,465	III	11	III
		13	III
		20	II x
		27	IV
		34	II x and IV
		40	II x and IV
2,477	II	6	II
		13	II b
		21	II b
		29	II b and IV
2,488	III	4	III
		14	IV
		21	IV
		28	IV
2,493	II	5	II
		15	III
		26	IV
2,497	I	2	I
2,519	II	9	II
		16	No pneumococcus.
2,521	II	20	" "
		27	" "
2,524	II	8	II
		15	II x
		22	No pneumococcus.
2,527	I	10	I
		24	No pneumococcus.
2,536	II	10	II
		17	No pneumococcus.
		25	" "
2,543	II	11	" "
		18	" "
		31	IV
2,547	II	20	No pneumococcus.
2,549	II a	19	" "
		26	" "
2,550	III	14	III
		21	IV

TABLE V—*Continued.*

Case No.	Type of pneumococcus during height of disease.	Day of disease.	Type of pneumococcus recovered.
2,551	I	6	I
		25	II a
		32	II a
2,553	I	17	No pneumococcus.
		24	" "
		31	" "
2,556	I	9	IV
		16	No pneumococcus.
2,557	I	10	IV
		17	No pneumococcus.
		24	II a
2,558	I	21	No pneumococcus.
		28	" "
2,560	I	11	IV
		18	II x
		25	No pneumococcus.
2,563	II a	12	IV
		26	No pneumococcus.
2,566	III	8	III
		15	III
2,571	I	9	No pneumococcus.
		16	IV
		23	IV
2,572	II	10	IV
		17	IV
		24	No pneumococcus.
		31	" "
		45	" "
2,573	II	19	" "
		26	" "
		40	II and IV
		47	IV
		57	IV
		64	No pneumococcus.
		71	II
		78	No pneumococcus.
		85	II
2,576	I	4	No pneumococcus.
		11	I
		18	No pneumococcus.

TABLE V—*Continued.*

Case No.	Type of pneumococcus during height of disease.	Day of disease.	Type of pneumococcus recovered.
2,615	II	7	II
		14	No pneumococcus.
		21	II
		28	II
2,622	II	16	II x
		23	IV
		32	II x
		39	II x
		53	No pneumococcus.
		60	II x
		72	II x
		79	II x
		86	II x
		100	No pneumococcus.
2,623	I	17	I
		24	No pneumococcus.
		31	I
		38	I
		45	I
2,625	I	8	I
		15	No pneumococcus.
2,626	II	11	II
		18	II
		25	II
2,630	I	3	I
		17	II x and IV
		24	II x
		38	II x
2,632	I	23	I and IV
		37	II x
2,633	I	4	I
		11	I
		19	I
2,689	III	7	III
		14	III
		21	III
		28	III
		35	III
2,647	I	6	No pneumococcus.
		13	" "
		20	I

TABLE V—*Continued.*

Case No.	Type of pneumococcus during height of disease.	Day of disease.	Type of pneumococcus recovered.
2,640	III	7	No pneumococcus.
		14	" "
		21	" "
		24	" "
		28	" "
2,581	II	4	II
		11	No pneumococcus.
		18	" "
		25	II
		32	No pneumococcus.
		47	" "
2,582	III	5	III
		12	III
		26	No pneumococcus.
2,586	II	8	" "
		22	" "
2,587	I	7	" "
		14	IV
		21	No pneumococcus.
		28	I
		35	No pneumococcus.
		42	" "
2,589	I	6	I
		12	I
		19	No pneumococcus.
		26	IV
2,593	III	3	III
2,595	I	16	No pneumococcus.
		23	II
		30	II
2,597	I	11	III and IV
		18	II x, III, and IV
		25	IV
2,602	I	12	IV
		18	IV
		25	IV
2,604	II a	12	III
		19	III
2,608	I	14	IV
		21	IV
		28	No pneumococcus.
		35	I

TABLE V—*Concluded.*

Case No.	Type of pneumococcus during height of disease.	Day of disease.	Type of pneumococcus recovered.
2,608	I	42	No pneumococcus.
		49	" "
		56	" "
		63	II x
		77	No pneumococcus.
		84	" "
2,611	II	2	II
		9	IV
		16	IV
		23	IV
		30	No pneumococcus.
		37	IV
2,652	I	13	I
		20	I
		27	I
		53	No pneumococcus.
		60	" "
		67	" "
		74	III
		81	No pneumococcus.
		88	" "
		95	" "

Table V records the results of a study of the length of time during which the type of pneumococcus responsible for the disease could be isolated from the sputum of 53 cases of lobar pneumonia due to Types I, II, and III. The carrier condition in the convalescent as measured from the onset of the disease is, on the whole, relatively brief.

The shortest period of carrying was 7 days, the longest 85 days. The long persistence of the disease strain may be associated with delayed resolution of the lung lesion or the occurrence of some complication. It is further evident from this study that the type organism may disappear or fail to be isolated from the mouth secretions of the recovered case at a given examination, and then be found again at a subsequent period. In only one instance during convalescence has a Type II pneumococcus been recovered from a patient convalescent from a Type I pneumonia. In this case the patient's wife

was a chronic carrier of the pneumococcus of Type II. A Type I pneumococcus has never been recovered from a patient convalescent from a Type II pneumonia.

DISCUSSION.

The results of the work detailed in the present report in general confirm the previous observations of Dochez and Avery on the relationship of the varieties of pneumococcus to lobar pneumonia and the occurrence of carriers of disease-producing types of pneumococci. Combination of the results of study over a period of years of the types of pneumococci inducing lobar pneumonia shows that the majority of instances of infection are due to organisms belonging to Types I and II. A small number of cases are due to infection with atypical pneumococci of Type II, as described by Avery, and of these organisms those belonging in the so called Subgroup II a are responsible for the greatest number of cases. Pneumococci of Type III in spite of their high virulence for human beings are responsible for the smallest percentage of cases of lobar pneumonia. The incidence of infection with pneumococci of Type IV seems to remain fairly constant from year to year. As the different types of pneumococci show differences in virulence for human beings, it is possible that variations in the mortality rate from year to year may be directly related to the seasonal dominance of different types.

Comparison of the pneumococci obtained from the mouth secretions of normal individuals with those isolated from diseased persons shows the existence of two general classes of organisms. One of these, consisting of pneumococci of Types I and II, occurs only in association with disease; the other, including Types III and IV, together with the atypical Type II organisms, causes a minority of the instances of disease, but includes the organisms which are commonly found in normal healthy mouths. In a small number of instances organisms of Types I and II have been found in mouth secretions of normal individuals. In all cases except two, however, in which this has occurred, it has been possible to trace direct contact of the individual with a case of lobar pneumonia caused by the same type of pneumococci. As a large number of normal persons have been examined, it is not surprising that in a few individuals in whom no

association with cases of lobar pneumonia could be traced organisms of the fixed Types I and II should have been discovered.

Among the types of pneumococcus which seem to adapt themselves readily to the normal mouth, Type III presents a paradox. This organism has the biologic characteristics that distinguish Type I and Type II from the other varieties of pneumococcus. All strains of these organisms with but few exceptions have common immunological characteristics. Although this organism is responsible for but a small percentage of the cases of lobar pneumonia, it produces an unusually severe type of the disease. Notwithstanding these facts, this organism is commonly met with in the mouth secretions of normal individuals where it may persist for long periods of time without producing pathological lesions. The strains of this group isolated from normal sputum possess immunological reactions identical with those of the strain isolated from cases of lobar pneumonia. It is of interest, however, that in a study of the mouth flora of cases of pneumonia due to *Pneumococcus mucosus* it has been found that this organism tends to disappear as rapidly from the mouths of patients as do the disease-producing Types I and II. The study of the varieties of pneumococci in the mouths of normal individuals and convalescents from lobar pneumonia shows that in such individuals the type of pneumococcus present may vary from time to time, and also that more than one type may be present at any given time. This suggests the possibility that the sputum types of pneumococci may be passed from one individual to another, and that occasionally an individual may acquire pneumococci of this class which possess sufficient pathogenicity to initiate disease.

These facts suggest the following conclusion concerning the epidemiology of lobar pneumonia. Infection with pneumococci of Type I and Type II must be regarded as dependent upon contact with a previous case of lobar pneumonia due to the same type of organism. These types of infection are either acquired by direct contact with an active case of pneumonia or by association with a healthy carrier of one of these types of pneumococcus, or with a convalescent from lobar pneumonia who still harbors in his mouth secretions the organism responsible for the disease. Infection with the sputum types of pneumococcus, namely, Types III and IV and the atypical strains

of Type II, may be autogenic in character, or due to the acquisition by the individual of a given race of one of these types to which he is especially susceptible.

SUMMARY.

1. Pneumococci of Type I and Type II are responsible for the majority of the cases of lobar pneumonia.

2. Among the pneumococci found in the mouths of healthy individuals, Type IV predominates, Type III is fairly frequent, and atypical organisms of Type II are occasionally encountered. Organisms of these types give rise to a minority of cases of lobar pneumonia.

3. Healthy persons intimately associated with cases of lobar pneumonia may harbor in their mouth secretions the highly parasitic pneumococci of Types I and II.

4. Occasionally a carrier of Type I or Type II pneumococcus is encountered in whom it is impossible to trace any contact with an infected patient.

5. Convalescents from lobar pneumonia may carry for a considerable period of time the type of pneumococcus with which they were infected.

THE VARIOUS FACTORS OF RESPIRATION IN PERSONS WITH PNEUMOTHORAX.

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PLATES 44 AND 45.

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In connection with the study of the respiration in experimental pneumonia, recently reported by Newburgh, Means, and Porter,[1] it became desirable to find out what effect a great reduction in the functioning lung surface would have on the various factors of respiration. Since pneumothorax presents this condition, the present research was undertaken. Our observations were made upon four cases in which pneumothorax was being produced for the treatment of pulmonary disease, and upon one case of spontaneous pneumothorax.[2]

The clinical data on the five cases are given below.

Case 1.—B. D., female, age 27 years. Artificial pneumothorax.

The first symptoms of pulmonary tuberculosis began 4 years ago. She lost ground rapidly for a year, in spite of rigid sanatarium treatment, and then injections of nitrogen were begun. She soon began to improve and the treatment has been continued ever since. There have been thirty-four injections in all. The dosage was about 1,000 cc. at first; now the average is 500 cc. In March, 1916, she had no symptoms of phthisis and was leading a normal life. Immediately after an injection she was somewhat short of breath, on exertion only.

Physical examination showed the usual signs of pneumothorax in the left chest. X-ray showed a nearly complete collapse of the left lung (Fig. 1).

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[1] Newburgh, L. H., Means, J. H., and Porter, W. T., *J. Exp. Med.*, 1916, xxiv, 583.

[2] Case 2 was a patient at the House of the Good Samaritan, Boston. We are indebted to the superintendent of that institution for making it possible for us to study the case and for furnishing much valuable information.

Case 2.—M. G., female, age 20 years. Artificial pneumothorax.

The first symptoms of pulmonary tuberculosis began $2\frac{1}{2}$ years ago. She has had sanatarium treatment most of the time since. 3 months ago she had much cough and sputum and was running a high evening temperature. Physical examination at that time showed extensive involvement of the left lung with cavity formation, and very slight signs at the right apex. Collapse of the left lung was then begun. Twelve injections of nitrogen have been made in all. Marked improvement was at once apparent. In November, 1915, physical examination showed signs of pneumothorax over the left chest, and x-ray showed an extensive collapse of the left lung.

Case 3.—S. M. H., female, age 32 years. Spontaneous pneumothorax.

The past history is negative.

10 months ago while dancing she suddenly became short of breath. 2 weeks later a diagnosis of pneumothorax was made. The dyspnea gradually decreased, and in November, 1915, she had slight dyspnea on exertion only. She entered the hospital on November 4, 1915, at which time physical examination showed the signs of pneumothorax over the whole left chest, and x-ray showed an extensive left-sided pneumothorax with slight displacement of the heart toward the right. The right lung showed evidence of tuberculosis.

The Wassermann test was negative. The blood showed slight secondary anemia. The pulse, temperature, and respiration rate were all normal while she was in the hospital.

Case 4.—M. P., female, age 22 years. Artificial pneumothorax.

The patient was believed to have a lung abscess.

The family and past history are unimportant. She entered the Massachusetts General Hospital on June 5, 1916, having previously been a private patient of one of us. The history was that of a productive cough of 7 months' duration with considerable loss of strength, and occasional night sweats. The sputum was purulent and copious and was raised most abundantly in the morning. There had been hemoptysis on several occasions. Physical examination at the time of entry showed an area of dullness in the middle of the left back with crackling râles and dullness, with increased breath sounds at the left top posteriorly.

The Wassermann test on the blood was negative. She occasionally ran a slight fever while in the ward.

An x-ray plate taken April 30, 1916, showed an area of dullness between the second and fourth ribs on the right, which extended across the chest from the lung root to the axillary border. This dullness was of an even consistency except at the borders where there was some mottling. The apices were clear, but there was some thickening about the hilus of each lung.

It was thought that she probably had a lung abscess in the lower part of the right upper lobe, and treatment by artificial pneumothorax was undertaken. The following injections of nitrogen were made while she was in the ward.

		cc.
June	8	600
"	9	500
"	12	650
"	18	900
"	24	300
July	3	700
"	6	800

A partial pneumothorax only was secured, as was shown by a series of x-ray plates (Figs. 2 and 3).

June 11. X-ray showed a small area of pneumothorax extending as high as the fifth rib in the axillary border on the right. Otherwise as previously described.

June 13. X-ray showed the lower lobe fairly well collapsed.

June 19. X-ray showed slightly more pneumothorax than at the last examination.

Case 5.—L. R., male, age 22 years. Artificial pneumothorax.

A case of bilateral pulmonary tuberculosis of 4 months' duration with active signs in the left upper lobe. Gradual loss of weight, fever, hemoptysis, and many tubercle bacilli in the sputum. As the disease was rapidly progressing and a brisk hemoptysis had set in, and since the patient was beginning to have an elevated temperature, it was decided to induce artificial pneumothorax and collapse the left lung (Fig. 4).

After the first two injections the patient raised about 8 ounces of blood during the succeeding 3 days, and it was feared the blood might come from the non-collapsed lung. As no signs of activity were noticeable in the non-collapsed lung, more nitrogen was introduced into the left pleural cavity, and soon after that the blood and sputum ceased.

At the present time the patient has a well collapsed left lung, is up and about every day, with no respiratory embarrassment, no cough or sputum, and has gained in weight.

The injections of nitrogen in the case were as follows:

		cc.
April	8	750
"	12	750
"	20	700
May	5	600
June	2	500
"	12	600

Observations with the Unit Apparatus.

Experiments with Benedict's Unit Apparatus (spirometer type, subjects breathing through a mouthpiece) were done with Cases 1, 2, and 3, in the fasting condition, lying prone, at complete rest. The data are given in Table I.

TABLE I.

Experiments with the Unit Apparatus.

Period.	Carbon dioxide per min.	Oxygen per min.	Respiratory quotient.	Ventilation per min. (reduced).	Respiration rate.	Volume per respiration (reduced).	Pulse.	Carbon dioxide in expired air (calculated).
	cc.	*cc.*		*liters*		*cc.*		*per cent*
Case 1. Weight 53.3 kg. Height 174.2 cm. Nov. 8, 1915.								
1	161	210	0.77	5.86	14.8	396	79	2.75
2	160	216	0.74	5.37	12.4	433	76	2.98
3	165	214	0.77	5.25	12.1	433	74	3.14
Average..	162	213	0.76					
Case 2. Weight 57.2 kg. Height 167.5 cm. Nov. 12, 1915.								
1	179	259	0.69	7.06	19.7	358	109	2.54
2	169	236	0.72	6.73	19.4	347	105	2.51
3	171	236	0.72	6.68	19.1	349	102	2.56
Average..	173	244	0.71					
Case 3. Weight 64.5 kg. Height 163.8 cm. Nov. 16, 1915.								
1	184	224	0.82	6.12	14.3	428	87	3.00
2	164	215	0.76	5.77	14.4	400	84	2.84
3	164	217	0.76	5.58	14.2	393	81	2.94
Average..	171	219	0.78					

Basal Metabolism.—This was calculated in each case from the average oxygen absorption and the calorific value of oxygen for the respiratory quotient obtained.[3]

The body surface of each subject was determined by the Du Bois height-weight chart,[4] and the metabolism expressed in terms of the area thus found, was as follows:

Case 1. 37.3 calories per sq. m. per hr.
" 2. 42.0 " " " " " "
" 3. 36.9 " " " " " "

[3] Williams, H. B., Riche, J. A., and Lusk, G., *J. Biol. Chem.*, 1912, xii, 357.
[4] Du Bois, D., and Du Bois, E. F., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 77.

Alveolar Carbon Dioxide Tension.—In three of the cases of artificial pneumothorax the alveolar carbon dioxide tension was determined by the Plesch-Higgins method. The results were:

	mm.
Case 1	44.3
" 2	39.0
" 5	41.5

Sensitivity of the Respiratory Center.—In three of the cases the sensitivity of the respiratory center was determined by obtaining the reaction to carbon dioxide. The experiment was carried out with the unit apparatus by replacing the absorbers with a large dead space, so that the carbon dioxide gradually accumulated. The curves shown in Tables II, III, and IV were obtained. In Case 4 one curve was secured before and two after the production of pneumothorax.

TABLE II.

Case 1. Reaction to Carbon Dioxide. Subject Lying on Back.

Carbon dioxide in inspired air.	Respiration rate.	Volume per respiration.	Total ventilation per min (unreduced).	Ventilation coefficient Increase in total ventilation. Start=100 per cent.
per cent		*cc.*	*liters*	*per cent*
		Feb. 5, 1916.		
0.00	12.0	460	5.51	100
1.42	14.3	481	6.90	125
3.06	13.0	605	7.88	143
3.63	15.3	611	9.36	170
4.66	15.7	765	12.00	217
5.30	15.8	870	13.75	250
5.81	17.0	876	14.90	270
6.50	18.2	975	17.75	322
		Mar. 27.		
0.00	15.5	403	6.25	100
2.58	15.8	536	8.49	136
3.71	16.0	596	9.55	153
4.66	17.5	667	11.69	187
5.70	19.0	741	14.10	226
6.41	19.3	920	17.75	284
7.14	20.0	910	18.20	292
7.60	21.0	1,017	21.35	341

TABLE III.

Case 4. Reaction to Carbon Dioxide. Subject Lying on Back.

Carbon dioxide in inspired air.	Respiration rate.	Volume per respiration.	Total ventilation per min. (unreduced).	Ventilation coefficient. Increase in total ventilation. Start=100 per cent.
per cent		*cc.*	*liters*	*per cent*
June 6, 1916. Before nitrogen injections had been begun.				
0.00	25.0	336	8.40	100
1.43	25.8	389	10.03	119
3.00	28.3	410	11.60	138
4.40	24.8	556	13.80	164
5.15	24.5	641	15.70	187
5.80	30.5	778	23.73	283
6.60	30.8	1,050	32.35	385
June 14. Has had three injections of nitrogen since last experiment.				
0.00	34.0	284	9.65	100
1.92	35.5	336	11.90	123
3.53	37.0	420	15.2	161
4.52	39.0	525	20.55	212
5.43	39.0	630	24.40	254
6.20	41.0	735	30.615	312
June 30. Two more injections of nitrogen.				
0.00	20.2	420	8.49	100
2.21	25.0	441	11.10	132
4.56	32.5	630	20.50	242
5.21	41.0	630	25.80	304
5.95	44.0	735	32.40	382

TABLE IV.

Case 5. Reaction to Carbon Dioxide. Subject Lying on Back.

Carbon dioxide in inspired air.	Respiration rate.	Volume per respiration.	Total ventilation per min. (unreduced).	Ventilation coefficient. Increase in total ventilation. Start=100 per cent.
per cent		*cc.*	*liters*	*per cent*
June 12, 1916.				
0.00	23.2	462	10.70	100
2.81	24.5	567	13.90	130
3.96	25.5	777	19.78	185
6.23	32.0	1,177	37.70	352
6.71	36.0	1,240	44.60	417

Vital Capacity.—The vital capacity was secured in Cases 1, 4, and 5. Case 1 fortunately had had some determinations made of her vital capacity while she was in college, which she was able to obtain for us. These determinations were as follows:

		liters
Oct. 1906	Vital capacity	2.6
" 1907	" "	2.5
	Weight 61 kg.	
" 1910	Vital capacity	2.1
	Weight 63 kg.	

The last was 2 years before the diagnosis of phthisis was made, and she says that she is sure she had tuberculosis at that time, as she distinctly remembers having a slight cough and tiring easily. This fact would explain the decrease in vital capacity.

On February 5, 1916, three determinations of her vital capacity, taken about 5 minutes apart, gave the following results:

	liters
Vital capacity	1.11
" "	1.11
" "	1.26

On March 27, 1916, the vital capacity was 1.5 liters.

The greater value obtained on March 27 is probably due to the fact that the last injection of nitrogen had been 4 weeks before, whereas on February 5, she had had one only 3 weeks before.

In Case 4 one determination was secured before any injections of nitrogen had been made, and two afterward.

On June 6 her vital capacity was 2.74 liters.

On June 14, after she had had three injections of nitrogen, it was 1.36, 1.40, average 1.38 liters.

On June 30, after five injections, it was 1.63 liters.

In other words, although by x-ray only a partial collapse of the right lung was present, yet functionally there was a reduction to one-half her original available ventilating space.

The vital capacity of Case 5 was observed but once. On June 12, it was 1.57 liters, which is less than half what we should expect in a man of medium weight and height.

DISCUSSION.

The observations on these five subjects show that all the factors of respiration are essentially normal in persons with one lung collapsed. The gaseous exchange is normal, for if we calculate the basal metabolism, we get normal values: 37.3 calories per sq. m. per hour for Case 1, 42.0 for Case 2, and 36.9 for Case 3 are all within 10 per cent of the normal average for women of their age. The respiratory quotients are perfectly reasonable except that that for Case 2, of 0.71, is somewhat low. She was, however, the subject with the most active tuberculosis and might easily have an increased catabolism and so reach a fasting respiratory quotient sooner than a normal subject.

The respiration rate, the volume per respiration, and the total ventilation of the lungs, in the three cases in which they were determined, are probably all within normal limits, though possibly the total ventilation in Case 2 is a little higher than is usually found.

The percentage of carbon dioxide in the expired air is within normal limits in Cases 1 and 3, but is somewhat low in Case 2.

The alveolar carbon dioxide by the Plesch method was normal in Case 1 (44.3 mm.) and Case 5 (41.5 mm.), but was somewhat low (39 mm.) in Case 2. This probably explains the low percentage of carbon dioxide in the expired air, and the higher ventilation in the case of the last subject. It may have been due to a very slight acidosis.

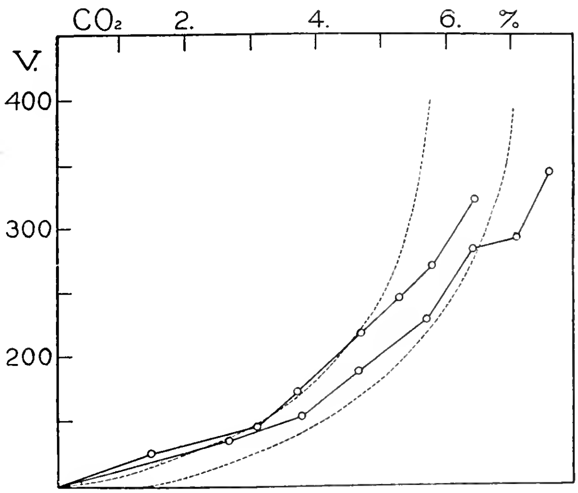
The reactions to increasing amounts of carbon dioxide in all the cases studied in this manner show that up to the point at which the respiration is trebled (at from 5.5 to 6.5 per cent of carbon dioxide in the inspired air) the increase in ventilation falls within the normal zone determined by Peabody,[5] as is shown in Table V. In this table the curves have been simplified by reducing the figures for ventilation increase to correspond to even percentages of carbon dioxide. This was done by plotting the actual observations and drawing the most probable curve through these points, then reading the values at the even percentages. The curves obtained with Case 1 are also shown graphically in Text-fig. 1.

[5] Peabody, F. W., *Arch. Int. Med.*, 1915, xvi, 851.

TABLE V.

Summary of Carbon Dioxide Reactions.

Subject.	Date.	Increase in ventilation to increasing amounts of carbon dioxide in the inspired air. Carbon dioxide.						
		1 per cent.	2 per cent.	3 per cent.	4 per cent.	5 per cent.	6 per cent.	7 per cent.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Case 1.	Feb. 5	116	131	143	190	235	285	
	Mar. 27	112	126	140	162	200	243	305
Case 4.	June 6	110	125	138	157	190	305	
	" 14	110	125	145	177	225	300	
	" 30	115	130	170	215	285	390	
Case 5.	June 12	112	122	140	190	263	336	455
Reactions of normal persons, taken from Peabody's diagram.		0 to 112	105 to 130	122 to 150	148 to 185	180 to 250	237 to 450	350 and up.



TEXT-FIG. 1. Case 1. Reaction to carbon dioxide. The ordinates show the percentage of carbon dioxide in the inspired air, the abscissæ the ventilation coefficient. The subject's normal ventilation, breathing atmospheric air, is called 100 per cent. At 200 per cent the ventilation is doubled, etc. The dotted lines represent Peabody's group of reactions in normal subjects.

The reaction obtained on February 5 with Case 1 was discontinued at 6.5 per cent of carbon dioxide; that of March 27, however, was continued up to 7.6 per cent carbon dioxide. It is interesting to note that in the latter curve the last two points show a distinct decrease in reaction, a flattening of the curve, while in Peabody's normal cases this was the point at which the curve became steeper. We interpret this finding as meaning that the subject reaches her limit at this point. The normal respiratory apparatus can increase the normal resting ventilation from four- to eightfold without great discomfort. This patient, with one lung, has a lower limit, but up to her limit reacts normally.

In some experiments upon one of us, Newburgh and Means found that the ventilation was trebled when the subject was doing about 500 kg. m. of work per minute.[6] This would be the equivalent in Case 1 of going up about three flights of stairs in 1 minute. As a matter of fact, she can do more than that without urgent dyspnea. At the end of each reaction to carbon dioxide, her volume per respiration was from 65 to 80 per cent of her vital capacity, but the rate had only increased in one experiment 50 per cent, and in the other 35 per cent. It would seem as though a further increase in the ventilation might have been met by an increase in rate, but such was, actually, not the case.

With Cases 4 and 5 much the same thing was found. The curves are both normal as far as they go. Case 4, with only a partial pneumothorax but with a vital capacity reduced to half of what it had been before the injection of nitrogen, was almost able to quadruple her ventilation. Case 5, with a more complete collapse and a vital capacity less than one-half what it should be, was more than able to quadruple his breathing. These functional capacities compare favorably with those of some normal persons.

The vital capacity was definitely reduced in the three cases of artificial pneumothorax, when it was determined.

[6] Means, J. H., and Newburgh, L. H., *J. Pharm. and Exp. Therap.*, 1915, vii, 454.

CONCLUSION.

It may be said that at rest all the factors of respiration, gaseous exchange, carbon dioxide tension, and the mechanical factors, are normal in persons with a collapsed lung, that the reaction to carbon dioxide is normal up to the point at which the respiration is trebled, or sometimes quadrupled, but that beyond that point a limit may be reached.

The ventilation of the lungs can be accomplished in an entirely normal manner in spite of a greatly reduced vital capacity. The only difference between normal persons and persons with a collapsed lung is that the latter when called upon to increase their ventilation reach their limit a little sooner than the former.

From these findings we might deduce that there will be no dyspnea except after moderate exertion. And this deduction is borne out by the histories of the patients.

In other words, in the lungs, as in other organs, there is a large factor of safety, one lung being as efficient as two, except when the work done calls for more than a threefold increase in the normal ventilation.

EXPLANATION OF PLATES.

Plate 44.

Fig. 1. Case 1. Artificial pneumothorax.
Fig. 2. Case 4. Before nitrogen injections were begun.

Plate 45.

Fig. 3. Case 4. Artificial pneumothorax.
Fig. 4. Case 5. Artificial pneumothorax.

THE COMPARATIVE RESISTANCE OF BACTERIA AND HUMAN TISSUE CELLS TO CERTAIN COMMON ANTISEPTICS.

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A number of chemicals are strongly bactericidal even in weak dilution when tested on bacteria suspended in broth cultures or in salt solution. In the presence of serum stronger solutions are usually necessary, while in order to kill pathogenic microorganisms growing in the tissues, as, for example, in infected wounds, the antiseptic must often be applied in such strength that body cells, as well as bacteria, are injured or destroyed.

An ideal antiseptic is obviously one that will kill the infecting agent without at the same time injuring body cells.

It is not practicable to carry out on infected wounds,—in man, at least,—experiments directed toward the discovery of such a substance. It occurred to the author, however, that in tissue cultures conditions might be made to approximate those in the living organism; for bacteria and tissue cells growing together *in vitro* may be easily subjected to the same chemical agents and the effect on each be directly observed.

Experiments were therefore undertaken to investigate the comparative resistance of body tissues (wandering cells and connective tissue cells) to various chemicals, including especially a number of those in common use as antiseptics.

Technique.

Human tissues were used throughout the experiments since it was thought that the results would be of more value if clearly applicable

to human beings. We have shown in a former paper[1] that connective tissue and wandering cells can be cultivated *in vitro* in a modified plasma medium almost as easily as similar tissues of lower animals. Tuberculous and Hodgkin's lymph glands removed at operation[2] and spleens taken out at autopsy a few hours after death were the tissues used. With each of these a migration of large mononuclear cells and connective tissue cells was obtained. The organism used was *Staphylococcus aureus*, chosen first because of the frequent infections caused by it, and, secondly, because it has been shown[3] to occupy a median position among the pathogenic bacteria in its resistance to disinfectants. It is more resistant, for example, than the streptococcus, but less resistant than *Bacillus pyocyaneus*. Staphylococci from two different sources were used; one strain from the throat of an apparently healthy individual, another from a case of furunculosis. The two strains exhibited little difference in their resistance to the chemicals tested.

In a few preliminary experiments the disinfectant or chemical to be tested was added in the desired strength to the plasma medium of the tissue culture. It was found, however, that certain of the substances used, especially iodine and hypochlorites, caused a liquefaction of the fibrin of the clotted plasma so that the cells found no framework upon which to grow and thus the experiment proved nothing as regards the effect of the chemical on cells. Furthermore, the results as regards the destruction of bacteria were also not uniform, probably owing to the imperfect diffusion of the disinfectant through the clotted medium. The limited diffusibility of chemicals through the plasma clot of tissue cultures has been well demonstrated by Rous.[4] It was therefore decided to subject the sterile and bacteria-laden tissues to the action of the disinfectants for a definite period before their incubation in tissue cultures. The tissues were cut into small

[1] Lambert, R. A., Technique of Cultivating Human Tissues *in Vitro*, *J. Exp. Med.*, 1916, xxiv, 367.

[2] I am indebted to the surgical staff of the hospital for their cooperation in supplying me with fresh tissue.

[3] Dakin, H. D., On the Use of Certain Antiseptic Substances in the Treatment of Infected Wounds, *Brit. Med. J.*, 1915, ii, 318.

[4] Rous, P., The Growth of Tissue in Acid Media, *J. Exp. Med.*, 1913, xviii, 183.

bits of a size suitable for culture preparations (about 0.5 mm. in diameter). These were washed quickly in one change of isotonic salt solution to remove the excess of blood and serum, and were then transferred to a dish containing a 24 hour broth culture of *Staphylococcus aureus* diluted five times with isotonic salt solution. The tissue fragments were allowed to soak for several minutes so as to become permeated by the bacteria. They were then transferred to dishes containing varying strengths of the chemicals to be tested. In these they remained for exactly 1 hour and were then removed to a dish of isotonic salt solution until put up in culture preparations,—usually a few minutes to half an hour. A second series was made with sterile tissues. Two sets of controls were prepared; one of untreated non-infected tissue and one of infected tissue. The tissue cultures were prepared as described in a previous paper,[1] using as a culture medium chick plasma and human serum in the proportions of 1 to 4. Five preparations were made for each strength of disinfectant used and for each of the controls. Besides the antiseptics in common use several other chemicals were tested, and the results are included in Table I. Many preliminary tests were necessary to approximate the lethal strengths of the chemicals for bacteria and cells. Since each experiment was repeated at least once the preparation of about 2,000 cultures was required.

DISCUSSION.

The table shows that in the case of the majority of the chemicals used (potassium cyanide, phenol, tricresol, hydrogen dioxide, and alcohol) tissue cells were definitely more easily killed than were bacteria. With certain other disinfectants the difference was not so striking. For example, in several experiments with mercuric chloride it was noted that in a few preparations there was a slight growth of connective tissue cells after exposure for 1 hour to a dilution of 1:20,000 or 1:40,000, a strength sufficient to kill or markedly inhibit the growth of staphylococci under similar conditions. It was observed, however, that the cells grew out from the centers of the tissue fragments, not appearing until after 4 to 5 days of incubation. It was concluded that growth in these cases was due simply to the

TABLE I.

Dilutions.	Mercuric chloride.		Potassium mercuric iodide.		Potassium cyanide.		Hypochlorites (Dakin's solution).		Iodine.		Phenol.		Tricresol.		Argyrol.		Hydrogen peroxide.		Glycerol.		Alcohol.	
	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Baceria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.	Cells.	Bacteria.
1 : 1																					0	0
1 : 5																			0	+	0	+
1 : 10																			±	+	+	+
1 : 20																			+	+		
1 : 50																	0	0				
1 : 100											0	0	0	0	0	0	0	±				
1 : 200											0	+	0	±	±	+	0	+				
1 : 500											+	+	0	+	+	+	±	+				
1 : 1,000							0	0	0	0	+	+	0	+								
1 : 1,500					0	+	0	±	±	0												
1 : 2,000					0	+	0	±	+	0±												
1 : 2,500			0	0	0	+			+	±												
1 : 5,000			0	±	0	+	±	+	+	+												
1 : 10,000	0	0	±	±	+	+	+	+														
1 : 20,000	0	±	+	+																		
1 : 40,000	±	±																				
1 : 80,000	±	+																				
1 : 120,000	+	+																				

0 = no growth; + = growth; ± = slight growth.

low penetrating power of mercuric chloride, for cultures in 1:80,000 never showed an active outgrowth of peripheral cells.

Alcohol in the strengths used (5, 10, 20, and 50 per cent) was found to be bactericidal in only the highest strength. On the other hand, it is noteworthy that human cells show no ill effects from exposure to 5 or 10 per cent alcohol for 1 hour. In one series noted there was indeed a better growth of the alcohol-treated tissues than of the controls. Further experiments, however, failed to demonstrate any definite stimulating action on the part of alcohol. The harmful effect of 20 per cent glycerol is probably referable to the partial desiccation of the tissues produced.

Iodine stands out as the one chemical tested to which cells were found to be more resistant than were staphylococci. A good growth of cells was seen after exposure to a 1:2,000 solution of iodine for 1 hour, a strength sufficient to sterilize the tissue completely in most instances.

These experiments afford further experimental evidence of the value of iodine as an antiseptic, and indicate that, at least in weak aqueous solution, it should not, as is often stated, injure or irritate the tissues.

It was observed, however, that iodine has the power of rapidly dissolving fibrin, a property, which, theoretically, should not be conducive to wound healing. A similar action by hypochlorites (Dakin's solution) was also noted. Although the wound-cleansing property of the latter, which evidently depends on this fibrin-dissolving function, is favorably emphasized by Dakin,[3] it would seem that the plastering together of wound surfaces by fibrin, which is thought to facilitate healing, would be prevented by the use on wounds of either iodine or the hypochlorites.

CONCLUSIONS.

The comparative resistance of bacteria and human tissue cells to antiseptics and other chemicals may be easily tested by tissue cultures under conditions which approximate those found in the living body.

A comparative study shows that while human cells (connective tissue and wandering cells) are highly resistant to many antiseptics,

they are in general more easily killed than bacteria (*Staphylococcus aureus*).

Of the antiseptics tested, which include mercuric chloride, iodine, potassium mercuric iodide, phenol, tricresol, hydrogen peroxide, hypochlorites (Dakin's solution), argyrol, and alcohol, the one which approaches most closely the ideal disinfectant is iodine, which kills bacteria in strengths that do not seriously injure connective tissue cells or wandering cells.

CHANGES IN PIGMENT EPITHELIUM CELLS AND IRIS PIGMENT CELLS OF RANA PIPIENS INDUCED BY CHANGES IN ENVIRONMENTAL CONDITIONS.

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PLATES 46 TO 48.

(Received for publication, June 10, 1916.)

One of the most important questions in the field of cell evolution is whether the fate of the cell is definitely determined as soon as it makes its appearance as an individual cell among the mass of embryonic cells of the developing body or organ, or whether the final type into which a cell will develop is determined by its environmental conditions, such as external physical and chemical factors. If the latter principle prevails during the process of the production of the organic form, the application of new external factors would create new types of cells not existing under ordinary natural conditions.

From recent experiments it has become evident that structures which we thought characteristic of certain species of cells are in no way specific for these cells but may also be assumed by other species of cells when brought under similar conditions. The above mentioned experiments may be divided into two groups, the first group comprising experiments in which an animal body served as the substratum for the development of the cells, while in the second group of experiments an artificial substratum was used.

The experiments of the first group have established three facts: (*a*) Embryonic cells of the same type may develop into different types as a result of their developing in different environments. Thus, primitive blood cells may develop into erythrocytes, if they become enclosed by the walls of blood vessels, or they may become granulated white blood cells, if they continue to develop outside the ves-

sels (1). More or less definite evidence was also obtained to show that certain relations exist between external factors and the various cell types into which the lymphoid hemocytoblasts outside the walls of the embryonic vessels finally develop (2). (*b*) Fully developed cells may undergo dedifferentiation into the embryonic state and then, if subjected to other conditions, may differentiate into another fully developed type of structure entirely different from the original. In the Nemertine *Lineus lacteus* Rathke, fully developed connective tissue cells (parenchymal cells) of small fragments including no part of the intestine, may dedifferentiate into a more primitive type of mesenchymal cell, the so called *Wanderzellen*, and then redifferentiate into the epithelial cells of the newly formed intestine (3). This circumstance also proves that even general structures characterizing large groups of cells, such as epithelium and connective tissue, are interchangeable and that a member of one group may be converted into an element of another group. (*c*) Highly differentiated cells may be directly converted into other highly specified cell types without dedifferentiation into the embryonic stage. This is true of the iris pigment cells which furnish the material for the regenerating lens after the loss of their pigment, after which they represent ordinary fully differentiated but unpigmented iris cells.

Through the discovery of tissue culture we have, so to speak, created a new type of body in which to grow a cell; *i.e.*, a new form of cell environment, in many respects different from the normal body that nature has given the cell in which to develop. None of the results of this group of experiments favor the view that specificity and constancy are fundamental features of cell evolution, for all cells, when compelled to grow within this new form of environment develop into cell types which, on the one hand are different from those existing in the animal body and, on the other hand, are characterized by a number of common structures, which designate them as tissue culture types. Champy (4), for instance, observed that the cells of four different organs—the kidney, the submaxillary, the parotid, and the thyroid of the rabbit—when cultivated outside the organism, change into one cell type; not only do the epithelium and connective tissue cells of one of these organs become indistinguishable from each other, but all the cells of all four organs become so much alike that it is no

longer possible to determine from which of these organs a given cell is derived. By applying different physical factors inside the substratum, such as a varying consistency of the medium and supporting surfaces, it has been proved that fundamental and general characteristics, typical of large groups of cells, such as the cuboid or cylindrical shape of the epithelium cells and the spindle shape of the connective tissue cells, become interchangeable.

From these facts it has been learned that we actually know very little about the developmental abilities of the body cells and the potentialities for differentiation they would exhibit if compelled to live under conditions different from those usually present within the organism. These facts more and more emphasized the part played by the external factors in cell development and led to the abandonment of the theory of cell specificity. Furthermore, it has become necessary to admit that our histological system, based upon certain characteristics which are assumed by the cell under conditions during the study of which this system was elaborated, has no more value than that of an artificial nomenclature, applicable to the organism only so long as it is studied under these particular conditions, and useful only as a means of gross orientation in the organism under the ordinary conditions. Through the relatively high constancy of these conditions in nature, we have been misled to believe that the types of our artificial system constitute the types of differentiation into which a certain cell must always develop, and that these cells develop into such types only because of a certain inherent structure which is present from the outset of the embryonic life of each cell; and, furthermore, that this structure, attached to the cell and unchangeable throughout its life, determines by its specificity the course of differentiation which characterizes this cell according to our artificial system of classification. As it has already been shown that many of the features thought to be specific can be changed by external factors, and that some of these changes are obviously operated and controlled by the laws of non-living matter, the question becomes more and more important as to what is produced in each cell by external factors, and what is due to an elementary structure —elementary in the sense that with its removal the cell would lose its attribute of cell.

As I (5) have been dealing with these problems in several recent papers I wish to present in the following short report another contribution to this subject and to describe some changes effected in explanted pigment epithelium cells and iris pigment cells, which I had the opportunity of observing during some work on these cells.

Material and Methods.

Fragments of iris and pigment epithelium from the adult leopard frog (*Rana pipiens*) were placed in hanging drops of a medium consisting, in a few instances, of two drops of plasma and one drop of muscle extract, but in most cases of two drops of plasma and one drop of aqueous humor. All the media were derived from the same species. The last described medium proved in many respects to be preferable to the first mentioned. In the first place it takes a long time to prepare the muscle extract, which in spite of all possible precautions sometimes becomes contaminated, whereas the aqueous humor (6), even when prepared in large quantities, requires only a short time and necessitates practically no special precautions in order to furnish a perfectly sterile medium. And, secondly, the coagulation effected by the plasma, when mixed with the aqueous humor, is much more satisfactory than the clotting of the ordinary muscle extract mixture and may sometimes last for several days.

EXPERIMENTAL.

Cells of iris and pigment epithelium when kept in the medium described above do not migrate as readily as do the cells of skin epithelium under the same conditions. Many fragments remained without signs of cell movement while under observation, that is to say, for several weeks, after which time they underwent degeneration. In most fragments from which cell migration took place we did not observe that the cells wandered out into the medium until from 2 to 6 days after explantation, while in cultures of skin epithelium this phenomenon was often visible as early as the night following explantation. The cells migrating into the medium are seldom as numerous in cultures of iris and pigment epithelium as in cultures of skin epithelium. Furthermore, we have not thus far been able to de-

tect any mitosis in the iris pigment cells and pigment epithelium cells when explanted.

Both types of cells are able to form membranes around the original fragments, the pigment epithelium cells producing membranes which resemble a typical epithelium more closely than the membranes formed from iris pigment cells. The membranes which are formed around fragments of iris are sometimes composed of iris pigment cells, unpigmented iris cells, and connective tissue cells (Fig. 1). In these membranes the unpigmented iris cells may be indistinguishable from the connective tissue cells.

As a rule, however, the iris pigment cells, as well as the pigment epithelium cells, leave the fragments without being grouped into membranes. They spread out from the edges of the fragment as single cells, or form very thin and delicate and sometimes branching chains of cells, or narrow processes ending in a very fine fimbriation formed by the elongation of each terminal cell into the medium. The last two forms are typical of the iris pigment cells.

Before reporting on the aspect which these cells assume when migrating into the medium, I wish to call attention to some of the chief features of the pigment epithelium cells and iris pigment cells under ordinary conditions.

The structures of the normal pigment epithelium cells are particularly characteristic, as many of them are not found in any other cells of the animal body. There are six characteristics which should be kept in mind when comparing these cells with the migrating pigment epithelium cells. (*a*) The cell body is differentiated into two distinct parts, one of which faces the choroid, with which it is in close contact. Its more or less homogeneous protoplasm contains the cell nucleus as well as several probably metaplasmatic structures, among which the yellow oil globules are present in almost every cell, and, as a rule, can also be seen in the migrating cells (Fig. 2). The other part of the cell, the so called pigment basis, faces the retina and consists mainly of a dense mass of rod-shaped, yellowish brown pigment granules. (*b*) These pigment granules are arranged irregularly, with no common direction of their long axis. (*c*) From the pigment basis many chains of pigment granules, the so called pigment strains numbering, according to Angelucci (7), up to 40, originate and radiate

toward the retina. As far as has been ascertained it appears that these chains are protoplasmatic processes containing the pigment granules. (*d*) The protoplasmatic part of the cell is partly covered by a firm cuticular cap, by means of which, as well as by means of a number of cuticular rods of fine arrow-like shape, the cells are fastened together. (*e*) Seen from the basal surface the pigment epithelium cells appear to be of hexagonal shape. (*f*) It has never been proved that these cells are capable of any kind of movement within the organism, either under normal or pathological conditions and in the numerous eyes which I have examined at different stages of development, degeneration, and regeneration I have never been ab'e to observe the slightest sign of movement on the part of these cells.

As far as this latter quality is concerned, the normal iris pigment cells entirely resemble the pigment epithelium cells, but in all other respects fundamental differences exist between these two species of cells. The iris pigment cells are not differentiated into two parts, but the spherical pigment granules with which the cell body is charged are distributed evenly throughout the whole cell. Pigment strains are lacking, all the cell boundaries being uniformly straight and unbroken. A simple basal membrane covers the basal surface instead of a highly differentiated cuticular cap. The shape of these cells is cuboid.

These same cells when migrating into the medium present a different appearance; the changes in the pigment epithelium cells are particularly conspicuous.

Figs. 2 and 3 show two pigment epithelium cells which have migrated into the medium and placed themselves at the edge of a membrane formed by these cells around the tissue fragments. The culture did not show cell migration until the 6th day. The cells shown in Figs. 2 and 3 were drawn on the 10th day after explantation, while in the act of isolating themselves. None of the pigment epithelium cells which are wandering out into the medium show the differentiation into a plasmatic part and a pigment basis which is so typical of the normal type of these cells. On the contrary, the cell body as shown in Figs. 2 and 3 is of the same structure throughout, the pigment being evenly distributed in the protoplasm. As the pigment granules are moved around slowly but continuously by the slow and

wave-like movements of the cell plasma they sometimes congregate more densely at certain points or may even occasionally be entirely shifted from one spot, leaving the cytoplasm free of pigment for a space of several minutes. The pigment granules no longer appear as an irregularly arranged mass, but most of them are now situated so that their long axis runs parallel to the long axis of the cell and to the direction of the cell medium. The most striking change is the complete disappearance of all the pigment strains. The cell border is smooth, and fringe-like protuberances are no longer visible.

Throughout all these changes the pigment epithelium cells have, in their appearance, approached much more closely the structure of a normal iris pigment cell. They no longer present typical differentiation into two parts and they have lost the pigment strains. This resemblance becomes still more marked if we compare the migrating pigment epithelium cells with the migrating iris pigment cells (8) instead of with normal ones, because the migrating iris pigment cells show the same slow movements of the pigment granules, by means of which several parts of the cytoplasm may become free of pigment for a short time.

From the changes observed thus far, it is evident that the cultivated pigment epithelium cells have lost all the features which characterize them in the organism as a result of the position which they there occupy between two essentially different elements, the retina and the choroid. In no cell hitherto examined in tissue cultures has the different action upon cell structure of various external factors been so conclusively demonstrated as in the pigment epithelium cells of the eye. While the effect of these various factors can be ascertained from an examination of the normal epithelium cells, the result of uniform environment is seen in the cultivated cell. As long as the cell is situated between two different media, each side shows a different structure; but as soon as it is surrounded by a uniform medium, a uniform structure must develop on all sides of the cell.

Besides the changes mentioned, other important differences between the cultivated and normal cells are visible. Both the pigment epithelium cells and the iris pigment cells lose all their individual structures. This is particularly conspicuous in the case of the former, because the cuticular cap of these cells is much more highly differ-

entiated than the simple basal membrane of the iris pigment cells and it produces a firm texture. The cuticular cap, as well as the basal membrane, has disappeared and thus both cells have become alike in that they lack cuticular structures. But in order to assume this character the pigment epithelium cell had to give up much more than the iris pigment cell, and this relation holds good for all other changes. Although this characteristic, *i.e.*, the lack of cuticular structure, is a feature common to all cultivated cells, it is particularly interesting in the present case, because the relation between the disappearance of this structure and the existence of uniform environment is most distinctly marked with regard to the pigment epithelium cell.

Finally we arrive at the shape and movement of both these cell types. Although they obey the ordinary rules which affect all cultivated cells and show, like the latter, a close relation between both phenomena, it appears to me of special significance that these cells behave in this respect like all other cells thus far examined in the tissue culture condition, because (*a*) a movement of these cells has never been observed within the body, while movements of most other cultivated cells, such as connective tissue, endothelial, and epithelial cells, commonly occur within the organs; and (*b*) the shape of the cultivated pigment cells is extraordinarily different from the original shape; in order to approach the usual shape of the free moving tissue culture cells, these cells must undergo enormous changes. This is shown in Figs. 4 to 10, which are drawn from cells moving in a semifirm (9) medium close to the cover-slip but without being attached thereto. Figs. 4, 5, and 6 show a single pigment epithelium cell, while Figs. 7 to 10 represent iris pigment cells. It will be seen that both cell types closely resemble each other also when in the condition of free movement. They are both spindle-shaped, like connective tissue cells, and show a similar disposal of the pigment. As a rule, the pigment is more dense in that part of the cell which lies backward of the direction of movement, than in the part flowing forward into the medium. In comparing Fig. 10 with a normal iris pigment cell the marked difference in shape is apparent.

In Figs. 4 to 6 the movement of one cell has been followed; this is seen to bear a close resemblance to the mode of movement of the epi-

thelium cells of the skin as described in a former paper (10). Fig. 4 was drawn at 3.30 p.m. while a long process at the rear was retracted into the cell and the pigment was accumulated into a dense pigment mass behind the nucleus. At 3.40 p.m. two processes at the front, which had already commenced to form at 3.30 p.m., were seen to be flowing out into two long pseudopodia, the space between which at 5 p.m. became more and more filled with the cytoplasm uniting both pseudopodia into one single process, the end of which was dichotomically branched (Fig. 6). The cell at the same time became narrower as a result of being elongated.

Although some important individual characteristics still persist in both cell types, nevertheless the changes already effected undoubtedly prove the importance of external factors in producing cell structures. Furthermore, it may be mentioned that prolonged cultivation under more favorable conditions may even change those structures which could not be influenced during the short time employed in our experiments. Attention should be called to the fact that all the changes described were established directly in the original cell and not gradually in cells derived from it by mitosis, for cell division was never observed to occur in these cultures.

The experiments seem to indicate that certain important and essential differences must exist between the retina and choroid in order to produce such peculiar structural differences of two sides of one cell. Further experiments may serve to throw some light upon the nature of these differences.

SUMMARY.

1. Retina pigment epithelium cells and iris pigment cells are placed in an identical medium.

2. The differentiation into two different parts, as well as the pigment strains of the migrating pigment epithelium cells, is lost. The normally highly specialized cuticular structures disappear. The cells begin to move and assume a spindle shape, thus losing their original hexagonal shape.

3. The migrating iris pigment cells begin to show a distribution of the pigment, similar to that exhibited by the migrating pigment

epithelium cells. The cuticular structures of this cell type also disappear. These cells also assume the ability of free movement and become spindle-shaped.

4. By means of these changes both cell types not only become more similar to the ordinary culture cell type, but also in certain respects, more like each other, whereas before explantation they were very unlike. In order to accomplish this the pigment epithelium cells must change much more thoroughly than the iris pigment cells.

5. The changes, particularly those of the pigment epithelium cells, show a definite relation to the changes of external factors, as they are the expression of the transmission from dissimilar conditions existing on different sides of the cells into conditions which are uniform on all sides.

In conclusion I wish to acknowledge the assistance of Mr. Nelson Anderson, who greatly facilitated this work.

BIBLIOGRAPHY.

1. Danchakoff, V., Über die Entwicklung des Blutes in den Blutbildungsorgan (Area vasculosa, Dottersackanhänge, Knochenmark, Thymus, Milz und lockeres Bindegewebe) bei *Tropidonotus natrix*, *Arch. mikr. Anat., 1te Abt.*, 1916, lxxxvii, 503, 577, 578; Concerning the Conception of Potentialities in the Embryonic Cells, *Anat. Rec.*, 1915–16, x, 415.
2. Danchakoff, Untersuchungen über die Entwicklung von Blut- und Bindegewebe bei Vögeln; das lockere Bindegewebe des Hühnchens im fetalen Leben, *Arch. mikr. Anat.*, 1908–09, lxxiii, 161; The Wandering Cells in the Loose Connective Tissue of the Bird and Their Origin, *Anat. Rec.*, 1916, x, 484.
3. Nusbaum, J., and Oxner, M., Fortgesetzte Studien über die Regeneration der Nemertinen. II. Regeneration des *Lineus lacteus* Rathke, *Arch. Entwcklngsmechn. Organ.*, 1912, xxxv, 236.
4. Champy, C., Dédifférentiation des tissus cultivés en dehors de l'organisme, *Bibliog. anat.*, 1913, xxxiii, 184.
5. Uhlenhuth, E., The Form of the Epithelium Cells in Cultures of Frog Skin, and Its Relation to the Consistency of the Medium, *J. Exp. Med.*, 1915, xxii, 76; Cultivation of the Skin Epithelium of the Adult Frog, *Rana pipiens*, *J. Exp. Med.*, 1914, xx, 614.
6. The preparation of the tissue fragments and method of taking blood and aqueous humor as we used to employ it, will be found described more extensively in, Uhlenhuth, Studien zur Linsenregeneration bei den Amphibien. I., *Arch. Entwcklngsmechn. Organ.* (in press).

7. Angelucci, A., Histologische Untersuchungen über das retinale Pigmentepithel der Wirbelthiere, *Arch. f. Physiol.*, 1878, 353.
8. For a picture see, Uhlenhuth, Studien zur Linsenregeneration bei den Amphibien. I., *Arch. Entwcklngsmechn. Organ.* (in press).
9. For the meaning of this term see, Uhlenhuth, The Form of the Epithelium Cells in Cultures of Frog Skin, and Its Relation to the Consistency of the Medium, *J. Exp. Med.*, 1915, xxii, 76.
10. Uhlenhuth, The Form of the Epithelium Cells in Cultures of Frog Skin, and Its Relation to the Consistency of the Medium, *J. Exp. Med.*, 1915, xxii, 86 and following pages, and Figs. 9 and 12.

EXPLANATION OF PLATES.

Subtract 18 mm. from the length of the tube given, in order to obtain its actual length. All the figures have been drawn at the level of the table instead of at the level of the microscope desk. The pictures are therefore on an average 32 per cent (linear) larger than when seen by the magnification of the lens system alone.

Plate 46.

Fig. 1. Iris and connective tissue cells forming a membrane around a fragment of the ventral part of the iris of *Rana pipiens*, which had been placed 4 days previously in two drops of plasma plus one drop of muscle extract of the same species. Drawn from the living specimen (Culture E 85), with the aid of an Abbé camera lucida. Leitz oc. 3; obj. 3.

Plate 47.

Figs. 2 and 3. Pigment epithelium cells of *Rana pipiens* migrating into the medium, drawn from the living specimen (Culture E 303), 10 days after the original fragment had been placed in two drops of plasma and one drop of aqueous humor of the same species. Abbé camera lucida; Leitz oc. 3; obj. $\frac{1}{12}$; oil immersion; tube 130.

Figs. 4, 5, and 6. An individual pigment epithelium cell of *Rana pipiens*, moving in two drops of plasma plus one drop of muscle extract (Fig. 4 at 3.30 p.m., Fig. 5 at 3.40 p.m., and Fig. 6 at 5 p.m.). Culture E 86. Abbé camera lucida; Leitz oc. 3; obj. $\frac{1}{12}$; oil immersion.

Plate 48.

Figs. 7, 8, 9, and 10. Four moving iris pigment cells in a semifirm medium. The original fragment (Culture E 276), a small piece of the dorsal part of the eyes, did not show cell movement until the 5th day. Figs. 7 and 8 were drawn on the 11th day after explantation; Figs. 9 and 10 on the 12th day after explantation. Abbé camera lucida; magnification of Figs 7, 8, and 9, Leitz oc. 3; obj. $\frac{1}{12}$; oil immersion; tube 130. Magnification of Fig. 10, Leitz oc. 3; obj. 6; tube 130.

700

THE SEPARATION OF SERUM INTO COAGULATIVE AND NON-COAGULATIVE FRACTIONS.

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(Received for publication, August 1, 1916.)

It is known that diphtheria antitoxin is associated in horse serum with its pseudoglobulin constituent. For therapeutic purposes this protein fraction has been extracted from the whole serum, thus obtaining a purer, or refined antitoxin preparation. It seemed possible that the same principle might be applied to the coagulative factor in serum, and that in this respect the active substance might likewise be linked with one protein fraction rather than with the serum as a whole. Horse serum is extensively used for hemostatic purposes, and if the coagulative principle could be separated, it might lead to the preparation of a refined hemostatic as potent as the original serum and containing a greatly diminished amount of protein. With this end in view, the albumin, the pseudoglobulin, and the euglobulin were separated by means of ammonium sulphate of various strengths and were tested for their coagulative efficiency. A brief report of this work was made some months ago.[1] Although this process does not yield protein fractions of absolute purity, it is the best method for the purpose, affording a sharp demarcation between the group of albumins and globulins, and a fairly sharp division between the soluble pseudoglobulin and the less soluble euglobulin.

The method followed was the one used in this laboratory for the preparation of refined diphtheria antitoxin. It consists of diluting the serum with one-half of its volume of water, and then precipitating the euglobulin with a 30 per cent ammonium sulphate solution; a small amount of pseudoglobulin comes down in the course of the process. The ammonium salt is then added up to 54 per cent to carry down

[1] Hess, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 125.

the pseudoglobulin, after which enough is added to the filtrate to precipitate all the albumin.[2]

TABLE I.

The Coagulative Effect of Adding the Protein Fractions of Human Serum to Normal Human Blood.

Control blood.	Euglobulin.		Albumin.		Pseudoglobulin.		Time.
	1 drop.	2 drops.	1 drop.	2 drops.	1 drop.	2 drops.	
							min.
–	–	–	–	–	–	–	2
+	+	+	+	+	+	+	4
++	++	++	++	++	++	++	8
++	+++	+++	++	++	++	++	10
++			++	++	++	++	12
+++			+++	++	++	+++	15
				+++	+++		18

Tests with Human Serum.

The nature of the tests and their results are shown in the tables. For the test illustrated in Table I, the fractions were prepared from human serum. It was determined in the course of later tests that the fractions had apparently identical action whether prepared from human, from horse, or from sheep serum; accordingly horse serum was used as it was most available. In view of the fact that it had been found that antitoxin is associated with the pseudoglobulin in the horse, but with the globulin in the serum of the goat, it was deemed advisable to carry out the preliminary tests upon human as well as upon animal sera.[3] It will be noted from Table I that the addition of one or two drops of euglobulin hastened coagulation, whereas the same amount of the other proteins failed to bring about this result. This was the usual course of all experiments of this kind, although in some instances the differences did not stand out so sharply.

[2] These separations were carried out by Mr. Edward Banzhof.

[3] Steinhardt, E., and Banzhof, E., unpublished work of the Research Laboratory of the Department of Health, New York.

TABLE II.

The Coagulative Effect of Adding the Protein Fractions of Human Serum to the Blood and Plasma of Hemophiliacs.

(A) Case 1. Blood.

Control blood.	Euglobulin.		Seroalbumin.		Pseudoglobulin.		Time.
	1 drop.	2 drops.	1 drop.	2 drops.	1 drop.	2 drops.	
							min.
–	–	–	–	–	–	–	4
–	+	+	–	–	–	+	8
++	++	+	–	–	+	+	12
++	++	++	+	+	+	+	14
++	++	++	++	++	++	++	18
++	+++	+++	++	++	++	++	20
++			++	++	++	++	24
++			++	++	++	++	28
++			+++	+++	++	++	34
++			+++		++	+++	36
+++					++		42

(B) Case 2. Plasma.

–	–	–	–	–	–	–	6
–	+	++	–	–	–	–	8
+	+	+++	+	+	+	+	10
+	++		+	+	++	+	12
+	+++		+	++	++	++	14
+			++	+++	++	++	16
++			+++		+++	+++	18
+++							26

Table II illustrates a similar test carried out in a case of hereditary hemophilia, where the blood had a coagulation time of 42 minutes and the plasma of 26 minutes.[4] Here again the euglobulin caused a marked shortening of the coagulation time, whereas the other fractions produced this effect to a less degree.

When it was not desired to obtain the three protein fractions separately but only to precipitate the euglobulin from the serum,

[4] For the coagulation tests of blood a simple method suggested by Howel (*Arch. Int. Med.*, 1914, xiii, 76) was employed. The basilic vein was punctured and 15 drops of blood were allowed to flow from the needle into a vial having a diameter of 1.5 cm. When the vial could be inverted without spilling any of its contents, coagulation was considered to be complete.

sodium chloride was employed instead of ammonium sulphate, as the former process is simpler and was found to give an equally effective product. The method pursued was as follows: The serum was saturated in the cold with chemically pure crystals of sodium chloride. It was then filtered, and a small amount of chloroform added to the precipitate, which was dialyzed for about a week. The dialysate was taken up with Ringer's solution, and 0.3 per cent of tricresol added. This was then filtered through a Berkefeld filter and bottled in 20 cc. vials.

The percentage of total solids, which may be regarded as euglobulin, varied in the different lots prepared, so that an estimation was made in every instance. In the lot used in the test shown in Table III, there was 2.5 per cent euglobulin, in another the percentage might

TABLE III.

The Action of Different Concentrations of Euglobulin on Purpuric and on Normal Plasma.

Purpuric Plasma.

Control plasma.	Euglobulin.		Time.
	1.25 per cent, 1 drop.	2.5 per cent, 1 drop.	
			min
−	−	−	2
+	+	+	4
+	++	++	8
++	+++	+++	10
++			14
++			18
++			22
+++			26

Normal Plasma.

Control plasma.	Euglobulin.				Time.
	1.25 per cent.		2.5 per cent.		
	1 drop.	2 drops.	1 drop.	2 drops.	
					min.
+	+	++	++	++	4
+	+++	+++	++	+++	6
++			+++		8
+++					10

be over 3 per cent, in which event it was diluted with Ringer's solution to 2 per cent. This test (Table III) shows that when we reach a certain maximum, the addition of a large amount of euglobulin ceases to have any further effect; that, for example, the coagulation time may remain the same whether we add a drop of a 1.25 per cent solution or of a 2.5 per cent solution of euglobulin to five drops of plasma. In another test (Table IV) made with a different preparation we note that two drops are more effective than one, but that we have then reached the optimal strength, and that three drops of

TABLE IV.

The Comparative Activity of Whole Serum and Euglobulin on Human Plasma.

Control plasma.	Whole serum.			Euglobulin, 2 per cent.			Time.
	1 drop.	2 drops.	3 drops.	1 drop.	2 drops.	3 drops.	
							min.
−	−	−	−	−	+	+	4
−	−	+	+	−	+	+	6
+	+	+	+	−	++	+	8
+	+	+	+	+	++	++	10
+	+	+	+	+	+++	+++	12
+	+	++	+	++			16
++	+	+++	+++	+++			20
+++	+++						22

euglobulin fail to heighten the effect. Table III likewise illustrates the fact that the euglobulin is able to hasten the clotting of plasma in purpura, another type of hemorrhagic disease. In this instance the blood was from a case of purpura showing all the usual symptoms—a slightly delayed coagulation time, a markedly diminished platelet count, a prolonged bleeding time, and a positive capillary resistance test.

Mode of Action of Euglobulin.

There are many ways in which substances exert a coagulative effect upon the blood. Without entering in detail upon this subject, it may be stated that we may divide these acceleratory substances into two groups; those which act by neutralizing inhibiting substances, and those which possess inherent activity in themselves.

To which class does euglobulin belong? The answer to this question can best be given by comparing the euglobulin to a substance which is known to derive its potency through overcoming the antithrombin of the blood. An experiment of this kind is reproduced in Table V, where the effect of tissue juice is compared with that of euglobulin.

TABLE V.

Comparison between the Neutralizing Power of Euglobulin and of a Thromboplastic Solution when Hirudin Was Added to Plasma.

(*A*) *Titration of Euglobulin and Thromboplastin Solution to Equal Strengths.*

Control plasma.	Euglobulin.		Thromboplastin, 1 drop.			Time
	1 drop.	2 drops.	1 : 1,250	1 : 2,500	1 : 5,000	*min*
+	+	+++	++	++	+	2
++	+++		+++	+++	++	3
++					+++	4
+++						6

(*B*) *Relative Coagulative Potency of the Solutions when Hirudin Was Added.*

Plasma + hirudin.*	Euglobulin, 1 drop + hirudin.	Thromboplastin, 1 : 2,500 + hirudin.	Time.
			min.
−	−	++	4
+	+	+++	6
+	+		8
+	+		10
+	++		35
+	+++		42
++			120
+++			130

* Hirudin in these tests refers to a 1 : 5,000 solution. This strength was found optimal for this experiment.

It has long been known that the various tissue juices of the body, those of the muscles, of the brain, of the glandular organs, etc., are powerful coagulants. It has also been established that they possess this power in virtue of being able to counteract the normal antithrombin of the blood. In this test thromboplastin was employed, which consists of a solution and suspension of brain, and has been

described elsewhere[5] as possessing marked coagulative power both *in vitro* and in its clinical application. In the first part of this table we see what might be termed a state of equilibrium between the two coagulants, a parallel test in which one drop of euglobulin and one drop of a dilution of thromboplastin (1: 2,500) hasten coagulation to the same degree. When, however, an antithrombic substance, a dilute solution of hirudin, is also added to the plasma, we note (Table V) a marked difference in action. The tissue juice almost completely neutralizes the hirudin, whereas the euglobulin is able only partly to overcome its inhibitory effect. It is evident therefore that these substances act in different ways, and that the euglobulin does not owe its power to a counteraction of the antithrombin. It is impossible to proceed further in this discussion, and to state the exact mode of action of euglobulin, as so many factors remain unknown as to the mechanism of coagulation. It would seem, however, in view of the fact that euglobulin is found in the normal blood, that it should be regarded as a physiologic coagulant, and its mode of action in these tests as merely an intensification of the normal clotting processes. In this connection we may add that this coagulative property is not common to globulins in general. For example, egg globulin showed no coagulative power (Table VI); and edestin, a vegetable

TABLE VI.

The Comparative Effect of Ovoglobulin and Ovoalbumin when Added to the Blood of a Hemophiliac.

Control blood.	Euglobulin.	Seroalbumin.	Ovoglobulin.	Ovoalbumin.	Time.
	1 drop.	1 drop.	1 drop.	1 drop.	
					min.
+	++	–	+	–	4
+	++	–	+	–	6
++	++	–	+	–	10
++	++	+	+	–	14
++	+++	+	+	–	16
++		+	+	+	18
++		+	++	++	20
+++		++	++	++	30
		+++	++	++	38
			+++	+++	52

[5] Hess, *J. Am. Med. Assn.*, 1915, lxiv, 1395.

globulin, when tested in various concentrations was found also to be completely devoid of activity. These negative results with ovo-globulin furthermore give evidence that the more rapid clotting, brought about by euglobulin, cannot be attributed to an increase in viscosity.

SUMMARY AND CONCLUSIONS.

The fact that the coagulative principle is closely associated with the euglobulin fraction of the blood is of clinical as well as of theoretical interest. It enables us to prepare a hemostatic containing about 2 per cent of protein which is more potent than the whole serum containing 6 to 7 per cent of protein. A preparation of this kind has been made in this laboratory from horse serum and employed during the past few months in numerous cases of bleeding. This euglobulin is absolutely sterile, as it has been passed through a Berkefeld filter, and is safeguarded against decomposition by the addition of 0.3 per cent tricresol. A detailed account of the therapeutic use of euglobulin will be reported elsewhere. It may be stated, however, that it has been employed in the various manifestations of intractable hemorrhage in which horse serum has been so largely resorted to of late years, and that in certain cases has seemed to bring about most satisfactory results; in no instance has there been any untoward effect. When intravenous injections are resorted to, euglobulin seems to be preferable to serum which contains fully three times the quantity of protein.[6] It also seems to be absorbed more quickly from the subcutaneous tissues. In all probability it will be found to meet the same indications as whole serum, possessing the advantages of concentration, and necessitating the introduction into the body of a much smaller amount of foreign protein.

[6] Refined diphtheria antitoxin is frequently used as a hemostatic when normal serum cannot be obtained. As this serum contains about 16 per cent of protein, which consists almost entirely of pseudoglobulin, it will readily be appreciated that it is not adapted to this use.

THE INFLUENCE OF CERTAIN FACTORS, ESPECIALLY EMOTIONAL DISTURBANCES, ON THE EPINEPHRIN CONTENT OF THE ADRENALS.*

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(Received for publication, July 31, 1916.)

In a study, mainly in cats, of the epinephrin discharge caused by electrical stimulation of the splanchnic nerves[1] we were led to consider the question whether the amount of epinephrin liberated by a given strength and duration of stimulation is related to the store of epinephrin already present in the adrenals, or is independent of that store. Desiring to compare the amount liberated in animals whose content was presumably high with the amount liberated in animals whose content was presumably low, we tried to exhaust the store, as far as possible, before the experiment on the liberation was made, by some of the procedures which according to Elliott[2] cause diminution of the epinephrin load. Morphine was tried and also β-tetrahydronaphthylamine, since Elliott states that these drugs produce marked exhaustion. But as in cats they also cause wide dilatation of the pupil, and we were using the eye reactions as one of the criteria of the presence of epinephrin in the blood, we eventually had recourse to frightening the cat by a dog. Elliott attributes the loss of epinephrin from the glands, induced by morphine and β-tetrahydronaphthylamine, to the fright occasioned in cats by these substances. But he did not make any experiments on the effect of actual fright, although he says that "the most direct

* A preliminary note was published in *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 184.

[1] Stewart, G. N., Rogoff, J. M., and Gibson, F. S., *J. Pharm. and Exp. Therap.*, 1916, viii, 205.

[2] Elliott, T. R., *J. Physiol.*, 1912, xliv, 374.

method of analysis would be that of inducing emotional fear at once, as by vexing the cat with a dog." He refers to the experiments of Cannon and de la Paz[3] as supplying evidençe of that nature, since "they showed that the emotion of anger or fear is associated with the appearance of adrenalin in the blood from a cat's suprarenal vein." We do not see that an increased liberation of epinephrin into the blood would necessarily imply a diminution in the epinephrin store of the glands. Elliott himself has given an instance of epinephrin liberation into the blood without any change in the store; namely, the liberation caused by electrical stimulation of the splanchnics. We shall produce evidence in another section of the paper that splanchnic stimulation can induce exhaustion of the epinephrin store, although far less easily than morphine, etc. It is, however, certain that a considerable amount of epinephrin can be discharged into the blood as a result of electrical stimulation of the splanchnics without any appreciable diminution being produced in the store. Indeed, the epinephrin passes into the blood at a more rapid rate than when procedures which distinctly exhaust the store (ether anesthesia, morphine, etc.) are employed. The load of epinephrin present at any given time in an adrenal would seem merely to represent the balance between formation and excretion, its absolute amount giving no index of the rapidity with which the epinephrin is built up and given off. Accordingly, even if it is assumed that fright causes increased liberation of epinephrin into the blood, the question still remains open whether the stock in the glands is diminished.

As a matter of fact, we did not find that in frightened cats the amount of epinephrin which could be excreted into the blood in response to splanchnic stimulation was less than in animals in which the experiments were made in the usual way without preliminary frightening. Nor did the assay of the epinephrin in the adrenals at the end of the experiment indicate that the stock had been appreciably exhausted by the frightening. This induced us to make some observations on the influence of emotional disturbances on the epinephrin content. The content was assayed by the method of Folin,

[3] Cannon, W. B., and de la Paz, D., *Am. J. Physiol.*, 1911, xxviii, 64.

Cannon, and Denis.[4] They compared the colorimetric method with the blood pressure method in pithed cats as described by Elliott and state that the two methods gave the same results. We made comparisons in two experiments and found also a sufficiently close agreement. We used Ringer extracts of adrenals and also acid extracts prepared according to the directions of Folin, Cannon, and Denis.

The colorimetric method proved to be satisfactory for the comparative estimations with which we were alone concerned. Where only small differences in the depth of the tint of the extracts of the two glands were present, we always checked the determinations with the standard by an observation in which the extracts of the two glands were directly compared with each other in the Duboscq colorimeter. The determination was always completed within 3 minutes, at most, after addition of the sodium carbonate solution.

Elliott states that animals killed without section of the nerve supply of one adrenal show equality of load in the two glands. We have seen abundant confirmation of this statement, although occasionally a difference well beyond the limits of error of the epinephrin assay exists, possibly more frequently in dogs than in cats (Table I). A similar equality was found in animals dying spontaneously, either of disease or after operations (Tables XIII and XIV) when the nerves of both glands were intact.

TABLE I.

Species.	Weight of adrenal.		Epinephrin.		Remarks.
	Left.	Right.	Left.	Right.	
	gm.	*gm.*	*mg.*	*mg.*	
Dog....	0.355	0.354	0.51	0.51	Killed with amyl nitrite and ether.
"	1.485	1.720	1.64	1.80	" by bleeding from carotids. Thyroid operation 24 hrs. previously.
Cat.....	0.238	0.231	0.30	0.26	Killed by chloroform.
"	0.241	0.227	0.30	0.30	Shot through head.

[4] Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 477.

TABLE II.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Duration of fright.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*	*days*	*hrs.*
Cat 1	0.261	0.224	0.28	0.22	5	5
" 2	0.326	0.250	0.24	0.25	5	5½
" 3	0.225	0.197	0.20	0.16	6	6
" 4	0.170	0.176	0.20	0.18	14	5½
" 5	0.210	0.221	0.25	0.24	10	5
" 6	0.165	0.188	0.14	0.15	33	5
" 7	0.154	0.156	0.16	0.18	33	5
" 8	0.200	0.196	0.16	0.15	33	5
Dog 1*	0.450	0.480	0.67	0.67	8	5
" 2†	0.625	0.621	1.00	0.92	5	5

* No. 1 was a small dog.

† No. 2 was a large male dog. Wound infected superficially. Infection did not extend into the abdomen.

Influence of Emotions on the Epinephrin Store of the Adrenals.

In Table II the results of eight experiments on cats and two on dogs are given. In the cats all the fibers coming to the left semilunar ganglion, including the major and minor splanchnics, had been divided according to Elliott's method. All operations were, of course, performed under ether anesthesia. Control experiments with morphine and with β-tetra showed that we invariably produced with these drugs the differential action on the two adrenals described by Elliott. Confirmatory evidence of the correctness of our technique was afforded by the marked differential effect observed in a number of the cats which died from various causes (Table XIV). It can, accordingly, be assumed with confidence that the innervation of the left adrenal in the cats used for the experiments was eliminated sufficiently to show definite differential exhaustion of the right, had the emotional disturbance been really associated with exhaustion.

Also, the length of time between the operation and the emotion experiment was sufficient to allow equality of load to be reestablished after the postoperative depletion of the unprotected adrenal, as was shown by control observations. For example, Cat 9 was killed suddenly 4 days after section of the fibers coming

to the left semilunar ganglion. The left adrenal weighed 0.330 gm. and contained 0.37 mg. of epinephrin; the right weighed 0.350 gm. and contained 0.37 mg. Cat 10 was suddenly killed 33 days after section of the fibers coming to the left semilunar ganglion. The left adrenal weighed 0.186 gm. and contained 0.17 mg. of epinephrin; the right weighed 0.200 gm. and contained 0.17 mg. of epinephrin.

The animals were subjected to emotional excitation for 5 to 6 hours by the presence of barking dogs. For this, the cats were enclosed in small cages so constructed that it was impossible for the dog to inflict physical injury upon the cat or to come in contact with it. The stimulation of ordinary sensory nerves was thus excluded as a factor. One of the observers personally took charge of the experiment from beginning to end. Of course, the emotions included fright, anger, fear, etc., the cats showing fight from time to time. The usual signs of sympathetic stimulation, dilatation of the pupil, erection of the hairs of the back and tail, etc., were naturally strongly elicited.

In some experiments dogs previously prepared by interference with the innervation of one adrenal were used to frighten prepared cats, two experiments on emotional disturbance being thus done with the same trouble as one. Control experiments with morphine (Table III) showed that the operations selected permitted a good differential effect, and this was confirmed in the case of prepared dogs dead of infections (pneumonia) (Table XIV).

In Dog 2 (Table II) the left major and minor splanchnics were cut in the abdomen. In Dog 1 the same nerves were divided, but, in addition, nerve strands coming towards the left adrenal from the lumbar sympathetic chain were severed.

It was shown by experiments with morphine that in dogs division of the major and minor splanchnics suffices to give a differential effect. In some of our animals, however, to be certain that enough of the innervation had been eliminated, we excised in addition the two lumbar ganglia next below the diaphragm and cut any strands seen coming from the lumbar sympathetic chain. In two of the animals, three lumbar ganglia were removed, including one above the diaphragm, which was perforated for the purpose. Not all these animals were employed for the observations on emotion; but to save repetition the operations used also for the morphine experiments, to be discussed in the next section, are given here. Some of the morphine experiments are mentioned in this section, since they serve as controls to show that the operations relied on were effective.

It was found that section of the major splanchnic only was not sufficient to give differential protection against exhaustion of the epinephrin store under morphine (Dog 9, Table III). Individual variations, of course, possibly exist in different dogs in this regard. In a dog (No. 17, Table XIV) dead of pneumonia, 13 days after division of the left major splanchnic, the right gland removed after death was found to be exhausted relatively to the left (0.31 mg. of epinephrin as

compared with 0.50 mg.). In another dog (No. 18) also dead of pneumonia, 14 days after section of the left major and minor splanchnics, excision of the two lumbar ganglia immediately below the diaphragm, and section of the strands going to the semilunar ganglion, marked protection of the load of the left adrenal as compared with the right was found (0.30 mg. in the left, 0.08 mg. in the right).

There is no doubt then, that the operations practised by us would have sufficed to reveal a relative exhaustion of the epinephrin store in the gland with the intact nerve supply under the influence of emotions, had such emotions been capable of causing exhaustion.

It will be seen from Table II that neither in the cats nor in the dogs is there any clear and constant deficiency in the epinephrin load of the right (still innervated) adrenal as compared with the left (denervated) gland. A difference of the same order of magnitude as that in Cat 1 may occasionally be seen in cats suddenly killed without operation (Table I). Our experiments yield no evidence that under emotional stress epinephrin is poured out into the blood in such quantities as to produce a decided impression upon the epinephrin store. In observations on the spontaneous liberation of epinephrin under experimental conditions,[5] we were struck by the steadiness rather than by the mobility of the rate of discharge. Attempts to produce acute changes in the rate in various ways were always without success. We were never fortunate enough to test adrenal vein blood at a moment when an outburst was taking place.

Whether experiments on the epinephrin content of the adrenals under the influence of emotional disturbance continued for a much longer time would yield a different result, we have, of course, no evidence. If the rate at which epinephrin passes into the blood were increased by fright it is conceivable that the rate at which it is built up, accelerated at first to keep the balance even, would eventually decline, thus permitting a deficiency in the load to be established.

Is the Morphine Depletion in Cats Due to Fright?—If emotion *per se* does not cause exhaustion of the epinephrin store the question at once arises whether the morphine effect in cats ought to be attributed to associated fright or to some other action of the drug. An easy way of testing this was afforded by the well known difference

[5] Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1916, viii, 479.

in the action of morphine upon cats and dogs. In dogs no signs of fright are, of course, produced. What happens, then, to the epinephrin load of the unprotected adrenal in dogs, as compared with that of the denervated gland, when morphine is administered? Elliott's result on morphinized cats is easily confirmed, but it is scarcely more difficult to prove that the same qualitative result is obtained on dogs. Accordingly we do not think that fright has anything to do with the morphine effect in cats.

TABLE III.

Morphine Effect on Epinephrin Store in Dogs.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Morphine.	Duration of morphine action.	Nerves cut.
	Left.	Right.	Left.	Right.				
	gm.	*gm.*	*mg.*	*mg.*	*days*	*mg.*	*hrs.*	
3	0.468	0.506	0.83	0.67	3	125*	7	Major and minor splanchnics.
4	0.430	0.384	0.67	0.37	4	125	8½	" " " "
5	0.280	0.284	0.36	0.23	10	150	8	" " " "
6	0.372	0.420	0.42	0.30	15	75	8	" " " " and 2 lumbar ganglia and chain excised below diaphragm.
7	0.550	0.565	0.30	0.21	11	80	8	Major and minor splanchnics, 2 ganglia below, and 1 above diaphragm.
8	0.711	0.754	0.97	0.98		150	7½	Control. No operation.
9	0.435	0.466	0.50	0.50	10	150	8	Major splanchnic only.
10	0.346	0.380	0.33	0.27	12	150	8	Only major splanchnic certainly cut.

* Morphine was given in two doses in all the animals, except No. 3, in which it was given in one dose.

Table III illustrates the results obtained with morphine in dogs. Remembering that section of the major splanchnic alone does not protect, or at least not constantly (Dogs 6 and 7), the reader will see that there is uniformly a definite deficiency of epinephrin in the right (innervated) gland as compared with the left (denervated) gland. A control animal (No. 8) morphinized for 7½ hours (without operation) had an equal load in the two adrenals.

TABLE IV.

Control Dogs Suddenly Killed.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Nerves cut.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*		
11	0.640	0.627	1.50	1.20	20 hrs.	Major and minor splanchnics.
12	0.400	0.408	0.72	0.72	9 days.	" " " "
13	0.640	0.550	0.83	0.83	4 "	" " " "

In Table IV results are shown on control dogs, with major and minor splanchnics cut, which were killed without being subjected to the action of morphine. The load in the two adrenals is seen to be equal, except in Dog 11, which was killed 20 hours after the operation. In this animal, as will be shown in the section on the postoperative deficit, the deficiency in the right (unprotected) adrenal no doubt represents merely the deficiency invariably seen after an operation and which has not been recouped in the short interval of 20 hours.

That signs which might be interpreted as those of fright are present in cats under morphine is, of course, not doubtful. Whether this interpretation is correct might be difficult to decide, and does not concern us here. It is, however, of interest to note that epinephrin seems to have nothing to do with those signs.

The signs of morphine fright can all be elicited by administering morphine to a cat in which one adrenal has been removed and the splanchnic supply of the other cut and in which accordingly no liberation of epinephrin through the splanchnics takes place. A cat in this condition behaves identically in the same way as a cat whose adrenal splanchnic supply has been cut on one side but left intact on the other. The pupils are widely dilated and there is the same characteristic restlessness and incessant movement. The content of epinephrin in the remaining adrenal of the first cat is found to be practically the same as that of the adrenal removed before the administration of morphine, while the content of the adrenal with intact splanchnic supply in the second cat is definitely diminished.

The dilatation of the pupil of the denervated eye, and the pilomotor effects associated with fright and anger were also observed

in cats after removal of one adrenal (right) and section of the nerves of the other, when the animals were frightened by a dog and in other ways.

Observations were made on four cats (Table V) in this way.

TABLE V.

Cats with the Right Adrenal Removed and Nerves of the Left Adrenal Cut.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*	*days*	
11	0.316	0.302	0.25	0.30	1	Died.
12	0.280	0.280	0.19	0.28	9	"
13	0.270	0.250	0.28	0.14	3	Killed by ether.
14	0.220	0.168	0.08*	0.18	37	" after experiments.

* The left adrenal was massaged before removal, in the course of an experiment.

They all yielded the same results. Sudden fright, as by hitting or jerking the holder, caused dilatation of both pupils instantaneously; that is, after an interval too short to be measured with a stop-watch and certainly far shorter than the interval required when reactions are evoked in a denervated eye by epinephrin. Frightening by a dog also caused good dilatation of the pupil on the side on which the superior cervical ganglion had been removed, as well as of the normal pupil. In certain animals the dilatation produced by fright was sometimes even greater than in the normal eye, although in other observations on the same animals it might be less. In other animals the dilatation although quite marked was never so great as in the normal eye.

When the animals were etherized the pupil of the denervated eye in every case dilated more widely than that of the normal eye. The same was true when a moderate degree of asphyxia was produced. In some of the animals repeated observations were made on the effect of fright, asphyxia, and etherization at different periods after the operation up to 5 weeks, always with the same result. In one of the cats (No. 14) an experiment was eventually made to determine whether any detectable amount of epinephrin was being given off in the blood of the adrenal vein, with an entirely negative result.[5]

No difference could be seen in the pupil and pilomotor reactions between these animals and control cats in which one superior cervical ganglion had been excised without interference with the adrenals.

In cats our observations on the effect of morphine upon the epinephrin store of the adrenals agree with Elliott's. For example, in Cat 15 the connections of the left semilunar ganglion were cut and 5 days later 50 mg. of morphine were injected. After 8 hours the cat was killed. The left adrenal weighed 0.212 gm. and contained 0.24 mg. of epinephrin. The right adrenal weighed 0.209 gm. and contained 0.14 mg. of epinephrin.

In interpreting the depletion of the store seen after the long continued action of morphine, ether, urethane, and other anesthetics the possibility must not be lost sight of that the rate of formation or of accumulation of epinephrin in the adrenal may be diminished by the drugs. This would cause depletion of the store if the rate of liberation continued unchanged, just as surely as an increased rate of discharge would cause depletion if the rate of formation remained the same.

Is the Depletion of the Epinephrin Store under the Influence of β-Tetrahydronaphthylamine in Cats Due to Fright?—Elliott has shown that in the cat β-tetrahydronaphthylamine causes marked depletion of the epinephrin store of an adrenal whose nerve supply is intact as compared with its fellow whose nerve supply has been previously severed. We can confirm this statement. For example, in Cat 16, 3 days after section of the nerve supply on the left side 3 cc. of a 2 per cent solution of the drug were injected. After 8 hours the cat was killed. The left adrenal weighed 0.240 gm. and contained 0.22 mg. of epinephrin. The right adrenal weighed 0.200 gm. and contained only a trace of epinephrin.

Elliott associates the exhaustion of the epinephrin store in the cat with the emotional alarm indicated by the behavior of the animal, the wide dilatation of the pupil, etc. Having found reason to doubt the interpretation of the morphine effect on cats as due to emotional disturbance, the obvious suggestion occurred to us to try the effect of β-tetra upon the epinephrin store in the rabbit, an animal in which, according to Mutch and Pembrey,[6] the symptoms "give the impression

[6] Mutch, N., and Pembrey, M. S., *J. Physiol.*, 1911, xliii, 109.

that the drug produces a state of increased psychic activity accompanied by muscular action appropriate to the emotions."

It did not prove easy, however, to devise an operation which gave with constancy a good differential effect on the two adrenals. According to Kahn[7] and to Nishi,[8] the right adrenal in the rabbit seems to derive from the left splanchnic a portion of the nerve supply concerned in changes in the epinephrin store and in the liberation of epinephrin. Nishi was led to this conclusion by investigations on the glycosuria and hyperglycemia caused by diuretin, and Kahn by the results of his experiments on the relation of the adrenals to puncture glycosuria.

We tried a number of different operations to see whether such a differential effect on the epinephrin store as that described in the section on the postoperative deficit, or as that caused by urethane, ether, and morphine in cats and dogs, or by β-tetra in cats could be produced.

TABLE VI.

Experiments on Rabbits in Which the Right Major and Minor Splanchnics Were Divided.

No. of animal.	Weight of adrenal.		Epinephrin.		Time between operation and death.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*		
1	0.176	0.152	0.16	0.14	14 days.	Killed without drugs.
2	0.160	0.160	0.10	0.10	11 "	" " "
3	0.362	0.300	0.23	0.23	7 hrs.	" " "
4	0.290	0.240	0.13	0.12	14 days.	3 cc. 2 per cent β-tetra, 8 hrs. before being killed.
5	0.250	0.224	0.11	0.10	11 "	5 cc. 2 per cent β-tetra, 7 hrs. before being killed.
6	0.175	0.180	0.10	0.11	14 "	50 mg. morphine, 8 hrs. before being killed.

Table VI shows the results of experiments in which the right major and minor splanchnics were divided in the abdomen. As will be seen, the results were negative. Even in Rabbit 3, which was killed 7 hours after the operation, the content was precisely the same in the two adrenals. In other words, there was no postoperative deficit in the left adrenal after section of the right major and minor

[7] Kahn, R. H., *Arch. ges. Physiol.*, 1911, cxl, 209.

[8] Nishi, M., *Arch. exp. Path. u. Pharm.*, 1909, lxi, 401.

TABLE VII.

Experiments on Rabbits in Which the Left Major and Minor Splanchnics Were Divided.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*		
7	0.390*	0.250	0.13	0.14	3 days.	Major and minor splanchnics cut. 50 mg. morphine, 8 hrs. before being killed.
8	0.280	0.270	0.11	0.10	1 day.	Major and minor splanchnics cut, and 1 ganglion near lumbar vein cut out. Died spontaneously.
9	0.400	0.280	0.20	0.13	14 days.	Major and minor splanchnics and strand from lumbar chain cut. 70 mg. morphine in 2 doses, 9 hrs. before being killed.
10	0.276	0.218	0.17	0.18	5 "	Major and minor splanchnics cut. 5 cc. 2 per cent β-tetra, 6 hrs. before being killed.
11	0.309	0.270	0.18	0.16	3 "	Major and minor splanchnics cut. 5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
12	0.296	0.270	0.11	0.11	11 "	Major and minor splanchnics torn from origin through diaphragm and first 2 ganglia below diaphragm excised. 5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
13	0.250	0.216	0.20	0.14	12 "	Major and minor splanchnics, 2 ganglia, and strand from chain cut. 5 cc. β-tetra, 6 hrs. before being killed.
14	0.300	0.300	0.20	0.20	14 "	Major and minor splanchnics and 1 strand cut. 5 cc. β-tetra, 9 hrs. before being killed.
15	0.220	0.180	0.18	0.16	14 "	Major and minor splanchnics and 2 ganglia cut. 5 cc. β-tetra, 8 hrs. before being killed.
16	0.200	0.161	0.16	0.15	3 "	Left semilunar ganglion excised. 5 cc. β-tetra, 8 hrs. before being killed.
17	0.116	0.104	0.11	0.10	½ hr.†	Major and minor splanchnics cut. Killed as control.

* Edema of left adrenal.

† From beginning of anesthesia till end of operation, 25 minutes. Animal killed ½ hr. after operation. The time is too short for a decided postoperative effect.

splanchnics, the operation affording no protection to the epinephrin store of the right gland as compared with that of the left.

The Epinephrin Store in Postoperative Edema of the Adrenal.—Division of the left major and minor splanchnics was next performed on a series of rabbits. Sometimes, in addition, strands seen coming from the lumbar chain were divided and lumbar ganglia excised. The results are shown in Table VII. They are complicated to a considerable extent by the fact that edema, to which the rabbit's adrenals seem to be susceptible after operations in their vicinity, developed in some of the experiments. This edema is associated with great depletion of the epinephrin store of the affected gland. After a time the edema disappears and the epinephrin reaccumulates. It is obvious that if observations, on the effect of morphine or of β-tetra for example, are made on an animal in which edema of the left adrenal is still present, a genuine diminution in the epinephrin content of the right gland may be completely masked. In spite of this complication, however, Table VII indicates that section of the left major and minor splanchnics does, in some rabbits at least, produce a real differential effect, in contrast to the entirely negative results on section of these nerves on the right side shown in Table VI.

Thus, in Rabbit 9, which received morphine 14 days after the operation the left adrenal contained 0.20 mg. and the right only 0.13 mg. It is practically certain that the other morphine experiment in the table (Rabbit 7) would have shown a similar result but for the edema of the left gland, which was marked. Control observations prove that a gland with this degree of edema, 3 days after operation, never contains nearly so much epinephrin as its fellow. There is, therefore, every reason to believe that before the morphine was given the content of the right adrenal in this animal was considerably higher than that of the left. Since after the morphine period there is practical equality in the two glands, a considerable depletion of the store must be assumed to have taken place. In the dog and cat we have not seen the occurrence of edema of an adrenal in consequence of an operation in its neighborhood. Nor was there any edema in the right adrenal of the rabbit in the experiments in which the right major and minor splanchnic nerves were severed, possibly because the nerves on the right side were divided somewhat farther from the gland than those on the left side and there was accordingly less risk of interference with the lymphatics of the right gland. It is also possible that the mere section of the nerves is a factor in the development of edema, and if the right adrenal in the rabbit derives part of its innervation from the left splanchnics, division of the right splanchnics alone would not be so likely to affect it.

We next tried division of the fibers between the left semilunar ganglion and the adrenal, combined with free separation of the gland from the surrounding connective tissue by passing a blunt dissecting instrument around the greater part of its circumference, in the hope of destroying most of the innervation of the left adrenal while leaving intact such part, if any, of the innervation of the right adrenal as may come from the left splanchnic. As expected, edema of the gland developed, with the concomitant decrease in the epinephrin content already alluded to, only the merest trace being sometimes found. When a sufficient interval was allowed to elapse the edema cleared up and the store of epinephrin was replenished. The results are shown in Table VIII. The marked postoperative deficit seen in Rabbit 22, killed 5 hours after the operation, shows that the left gland was well protected relatively to the right by the operation. Edema of the left had not had time to develop in the few hours which had elapsed. In Rabbit 21, killed half an hour after the operation, no postoperative deficit was shown, the time being too short. This indicates that the marked depletion of the stock of epinephrin through the splanchnic nerves which follows surgical operations develops gradually. A number of the animals were killed as controls to determine the time necessary for recuperation of the epinephrin store.

In 7 days (Rabbit 18) the content of the left adrenal was not yet equal to that of the right. In 4 days (Rabbit 20) the depletion associated with the edema had reached its maximum, only a trace of epinephrin being present in the left adrenal. In 1 day (Rabbit 19), on the other hand, the left adrenal contained if anything, rather more than the right, the postoperative effect on the right (innervated) gland in all probability having not yet entirely disappeared, and the edema effect on the left not having attained its maximum, as is indicated by the relatively small excess of weight of the left gland. In Rabbit 28, which, like Rabbit 20, showed marked edema of the left gland after 4 days, no epinephrin reaction whatever was obtained from that gland.

Such observations as were made with β-tetra and with morphine and other drugs on animals prepared in this way did not yield decisive results for our immediate purpose, as, in the absence of a sufficient number of control animals kept for several weeks, it is not possible to be sure that equality in the epinephrin content of the two glands had been reached before the drugs were administered.

The results, however, seem worthy of being recorded because of the way in which they illustrate the depletion of the epinephrin store

TABLE VIII.

Experiments on Rabbits in Which the Nerves between the Left Semilunar Ganglion and the Gland Were Divided and the Gland Was Separated from the Surrounding Tissue.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	gm.	mg.	mg.		
18	0.304	0.173	0.22	0.33	7 days.	Killed without drugs.
19	0.170	0.134	0.13	0.11	1 day.	" " "
20	0.440	0.210	Trace.	0.24	4 days.	" " "
21	0.252	0.210	0.24	0.27	½ hr.*	" " "
22	0.140	0.146	0.11	Trace.	5 hrs.	" " "
23	0.154	0.120	0.12	0.12	15 days.	3 gm. urethane 6½ hrs. before being killed.
24	0.175	0.156	0.11	0.11	17 "	Ether for 2½ hrs. Died of ether.
25	0.390	0.330	0.20	0.15	4 "	5 cc. 2 per cent β-tetra. Died in 6 hrs.
26	0.180	0.122	0.14	0.12	7 "	3 cc. 2 per cent β-tetra, 8 hrs. before being killed.
27	0.190	0.152	0.21	0.21	14 "	3 cc. 2 per cent β-tetra, 8 hrs. before being killed.
28	0.310	0.200	0.00	0.11	4 "	50 mg. morphine, 8½ hrs. before being killed.
29	0.326	0.228	0.14	0.24	7 "	50 mg. morphine, 6 hrs. before being killed.

* From beginning of anesthesia till end of operation was ½ hr. Animal killed ½ hr. after operation.

by conditions leading to edema of the glands after complete or at least extensive section of their secretory nerves and the rate of recuperation of the store of the denervated glands. It is probable that if the animals were kept longer the operation could be used to make tests for differential effects on the two adrenals.

Finally we tried division of the nerves between the semilunar ganglion and the left adrenal, with, in addition, section of any strands seen coming from the lumbar sympathetic towards the adrenal and excision of one or two of the sympathetic ganglia next below the diaphragm. Care was taken to avoid, as far as possible, interference with the gland. This operation gave the best results as regards constancy of effect, although even here the marked differences so easily obtained in dogs and cats were not seen.

TABLE IX.

Experiments on Rabbits, in All of Which the Nerves Were Divided between the Left Semilunar Ganglion and the Adrenal without Disturbance of the Gland. In Some of the Animals Portions of the Lumbar Sympathetic Chain Were Also Cut or Excised.

No. of animal.	Weight of adrenal.		Epinephrin.		Time after operation.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*		
30	0.324	0.276	0.18	0.14	14 days.	5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
31	0.300	0.260	0.24	0.20	10 "	5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
32	0.188	0.155	0.15	0.13	9 "	2 ganglia nearest diaphragm excised. 5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
33	0.140*	0.103	0.08	0.08	21 "	Strands crossing aorta cut. 5 cc. 2 per cent β-tetra, 8½ hrs. before being killed.
34	0.380	0.400	0.22	0.22	20 "	1 ganglion just below diaphragm excised. 5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
35	0.220	0.182	0.20	0.16	11 "	1 ganglion just below diaphragm excised. 5 cc. 2 per cent β-tetra, 8 hrs. before being killed.
36	0.356	0.314	0.20	0.16	8½ hrs.	Strands crossing aorta cut. Killed as control.

* In Rabbit 33 the left adrenal was found bound down by firm adhesions.

Table IX illustrates the findings. In Rabbit 36, killed to determine whether a postoperative deficit had been established in the right (innervated) gland, a positive result was obtained, although the difference was by no means as large as is often found in rabbits when one adrenal is excised and the other with its innervation intact left in for several hours longer (Table XI).

A number of experiments were made with β-tetra on animals prepared in this way. While in most cases some deficiency in the epinephrin content of the still innervated gland was found after the action of β-tetra, it was never nearly so great as in cats.

In Rabbit 33 the low content (0.08 mg.) in both adrenals is worth noting. The left gland was considerably larger than the right and was still, although 3 weeks

had elapsed since the operation, bound down by adhesions. It is probable, therefore, that the epinephrin store had not yet reached the initial amount. If so, since the right gland also contained only 0.08 mg. after β-tetra had acted for 8½ hours, the drug must have caused some depletion in the unprotected right adrenal. In cats after Elliott's operation on the connections of the semilunar ganglion, we have not seen any edema, and, as he states, equality of the epinephrin content of the two glands is soon established. In one cat (No. 17), however, which was killed as a control, 11 days after the operation on the nerves of the left adrenal, the gland was found firmly bound down by adhesions and the epinephrin content was decidedly less than that of its fellow. The left adrenal weighed 0.398 gm. and contained 0.22 mg. of epinephrin. The right adrenal weighed 0.371 gm. and contained 0.33 mg. of epinephrin.

This is a very exceptional result and was probably due to the fact that we happened to cut a vein in the vicinity, and in securing it some dissection was needed. Such an experiment would, of course, be rejected as a control.

As regards the question raised concerning the way in which β-tetra causes exhaustion of the epinephrin store in cats, it is evident that these observations do not lead us to the same definite conclusion as in the question concerning the mode of action of morphine. The results, indeed, might be interpreted as in favor of Elliott's view that the emotional disturbance in the cat is the efficient factor, since signs of such disturbance are less marked in the rabbit.

It must be kept in mind, however, that the rabbit has a more limited range in the expression of emotion than the cat, and β-tetra certainly produces excitation of sympathetic and other mechanisms which may be concerned in this expression. The pupil is widely dilated, the pulse and especially the respiration are markedly accelerated. There is cutaneous vasoconstriction, the ears being distinctly cool and the ear vessels narrowed. Usually the animals show restlessness and sometimes stamp with the hind feet. The rectal temperature in our observations rose 0.7–1°C.

The contrast between a rabbit under morphine and a rabbit which has received β-tetra is great. Yet it is easier to demonstrate diminution of the epinephrin store in the rabbit by morphine than by β-tetra. While our observations on morphine seem to indicate clearly that fright is not an essential factor in the morphine depletion, it would be unwarranted to claim that our observations on β-tetra show that fright is not the cause of the epinephrin depletion, so convincingly demonstrated by Elliott in the cat, under the influence of that drug.

It may be permissible to point out, however, that one drug (morphine) which causes different emotional phenomena in the cat and dog produces the same effect on the epinephrin store, while another drug (β-tetra) which causes in the cat and rabbit emotional effects of the same general quality and, perhaps, if the difference in the emotional development of the two animals is taken into account, not so very unequal in degree, affects the epinephrin store very differently.

Comparison of the Epinephrin Load of the Two Adrenals in Rabbits when Removed at Different Times.

Some experiments on rabbits were made in which the left adrenal was first excised, and the right subsequently, after varying periods of time. The results are shown in Table X. It will be seen that in the five animals killed without drugs (Nos. 37, 38, 39, 40, and 41) the right adrenal contains more epinephrin than the left contained at the time of excision. Where the difference is small it could be accounted for

TABLE X.

Experiments on Rabbits to Determine the Load of the Right Adrenal at Varying Intervals after Removal of the Left.

No of animal.	Weight of adrenal.		Epinephrin.		Interval between excision of 2 adrenals.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*	*days*	
37	0.454	0.470	0.22	0.28	14	Killed without drugs.
38	0.162	0.190	0.10	0.13	14	" " "
39	0.312	0.324	0.14	0.22	17	" " "
40	0.420	0.346	0.16	0.33	26	" " "
41	0.270	0.176	0.13	0.17	18	Abscess at site of operation, but superficial. Killed without drugs.
42	0.158	0.164	0.16	0.16	16	50 mg. morphine, 7 hrs. before removal of right adrenal.
43	0.450	0.640	0.22	0.47	16	5 cc. 2 per cent β-tetra, 9 hrs. before removal of right adrenal.
44	0.288	0.350	0.18	0.33	16	5 cc. 2 per cent β-tetra, 9 hrs. before removal of right adrenal.

by the diminution of the epinephrin load in both adrenals even during the short operation necessary for removal of the left adrenal. But such a difference as that seen in Rabbit 40, in which the interval between the removal of the two glands was greatest is too large to be explained in this way. In Rabbit 44 this factor was taken into account by removing the second adrenal by an operation which in duration and technique as nearly as possible duplicated the first operation. It must, therefore, be concluded that the load of the remaining adrenal in most of the animals is increased above its amount at the time of excision of the first gland. More observations, especially with longer intervals, would be necessary to make sure that the remaining adrenal can accumulate more epinephrin than is ever seen in one adrenal when both are present. A glance at the other tables which give results on rabbits will show that the number of high loads of the right adrenal in Table X is unusually great for the number of animals, and this is suggestive.

The experiments were originally undertaken with the idea that if the right adrenal regained only its initial content after the operation the animals could be employed for testing the action of substances like morphine or β-tetra on the epinephrin store. The fact that the load increased so much rendered this method too uncertain. The difference in the result in Rabbit 42 which received morphine, and Rabbits 43 and 44 which received β-tetra, is nevertheless striking. The preponderance of the content of the right adrenal in the last two experiments as compared with the left indicates strongly that the influence of β-tetra in discharging the right adrenal was at any rate not great. On the other hand, unless Rabbit 42 was the single exception among the eight animals in the table, some discharge of the right adrenal must have been occasioned by the morphine, since the content after morphine was just the same as that of the left adrenal.

Postoperative Depletion of the Epinephrin Store.

The fact has already been alluded to that during and for some time after operations a more or less progressive decline takes place in the epinephrin store of an adrenal whose nerve supply is intact as compared with its denervated fellow. The phenomenon was well illustrated in experiments in which one adrenal was removed, and then after varying intervals of time the other. The exact opposite of the results shown in Table X is then seen; that is, a marked deficiency in the second adrenal as compared with the first. For example, in

Rabbit 45 (Table XI) the left adrenal was excised and contained 0.37 mg. of epinephrin. The wound was sewed up, the whole operation lasting 20 minutes, and after 8½ hours the right adrenal was removed. It contained only 0.13 mg. of epinephrin.

TABLE XI.

Experiments Showing Postoperative Depletion of Epinephrin Store of One Adrenal as Compared with the Other Removed at Operation.

No. of animal.	Weight of adrenal.		Epinephrin.		Interval between excision of 2 adrenals.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*		
Rabbit 45......	0.394	0.384	0.37	0.13	8½ hrs.	
Cat 18.........	0.220	0.218	0.18	0.14	5 "	
" 19.........	0.205	0.238	0.22	0.25	2 days.	
Rabbit 46......	0.318	0.314	0.20	0.11	6½ hrs.	5 cc. 2 per cent β-tetra, 6½ hrs. before being killed.
" 47......	0.092	0.084	0.14	0.08	7 "	50 mg. morphine, 7½ hrs. before being killed.
" 48......	0.267	0.238	0.11	0.09	6½ "	50 mg. morphine, 6½ hrs. before being killed.
" 49......	0.380	0.408	0.28	0.12+	8 "	50 mg. morphine, 8 hrs. before being killed.
Cat 20.........	0.240	0.300	0.33	0.20	6 "	50 mg. morphine, 6 hrs. before being killed.
" 21.........	0.225	0.258	0.22	0.14	7 "	50 mg. morphine, 7 hrs. before being killed.
" 22.........	0.230	0.221	0.26	0.26	8 "	Right adrenal excised and nerves of left adrenal cut. 50 mg. morphine 8 hrs. before being killed.
Dog. 14........	0.354	0.453	0.67	0.42	8 "	175 mg. morphine 8 hrs. before being killed.
" 15........	0.352	0.360	0.38	0.30	7 "	200 mg. morphine 7 hrs. before being killed.

This great depletion was assuredly not present at the end of the operation, as shown in other experiments. Etherization for 20 or 30 minutes does not produce a great impression on the store of an adrenal removed at the end of that time. Unless, then, the depletion

is an after-effect of the short etherization, which does not seem likely, it must be due to some other factor, probably the sensory stimulation acting during the postoperative period.

Similar results in animals with one gland protected by section of its nerves have already been given in other tables; for example, Rabbit 22 (Table VIII). 5 hours after section of the nerves of the left adrenal, the animal was killed. The right adrenal contained only a trace of epinephrin, the left 0.11 mg. In the same table and in Table VII examples are given where in half an hour after the operation practically no postoperative difference was present.

The recuperation of the store is usually evident in 1 day, although it may not then be complete. For instance, in Cat 19 (Table XI), 2 days after removal of the left adrenal the right contained 0.25 mg. as compared with 0.22 mg. in the left. Probably the difference represents about the amount by which the store of the left adrenal was diminished during the short operation for its removal. Many of the animals received morphine immediately after excision of the first adrenal (always the left except in Cat 22), and one (Rabbit 46) received β-tetra. The effect, if any, of these drugs is complicated by the postoperative depletion.

Cat 22 shows how completely section of the nerve supply of an adrenal protects the store even against the combined effect of the operation and morphine. All preparations having been made to insure almost simultaneous removal of one adrenal and denervation of its fellow, the right adrenal was excised in 5 minutes, and the nerves of the left adrenal were cut within the next 2 minutes. Even after 8 hours, the epinephrin content of the left adrenal was precisely the same as that of the right; namely, 0.26 mg.

In Table XII are given the results of assays of the adrenals in a number of experiments on the liberation of epinephrin into the blood.[5] All except the first show a relatively low content, since the nerves were cut in the course of the experiment, usually some hours after the beginning. Where the innervation of one adrenal is divided after several hours of experimentation, so great a depletion of the epinephrin store of both glands has already taken place that subsequent division of the nerves of the other, an hour or two later, does not generally establish such a striking difference between the two glands as when the nerves on one side are severed at the beginning.

In Table XIII are shown the epinephrin assays of the adrenals obtained in other experiments on liberation of epinephrin. In these the innervation of the glands was not interfered with. Equality of content in the two adrenals of the same animal is well shown. While there are, of course, variations in the different animals, the average content is lower than that of the cats in Tables I and II.

TABLE XII.

Depletion of the Epinephrin Store of the Innervated Adrenal during Physiological Experiments.

No. of animal.	Weight of adrenal.		Epinephrin.		Duration of experiment.	Remarks.
	Left.	Right.	Left.	Right.		
	gm.	*gm.*	*mg.*	*mg.*	*hrs.*	
Cat 23	0.260	0.286	0.18	0.25	5	Connections of right semilunar ganglion cut 11 days before experiment.
" 24	0.230	0.230	0.17	0.28	$2\frac{1}{2}$	Connections of right semilunar ganglion cut 15 days before experiment. Used for liberation experiment and died during the experiment. In poor condition; abortion the day before.
" 25	0.204	0.211	0.10	0.17	5	Right major and minor splanchnics cut $3\frac{1}{2}$ hrs. after beginning of experiment.
" 26	0.197	0.201	0.14	0.16	$5\frac{1}{2}$	Right sympathetic (in thorax) cut $2\frac{1}{2}$ hrs. after beginning of experiment. Left sympathetic cut $2\frac{1}{4}$ hrs. later.
" 27	0.140	0.140	0.11	0.11	$3\frac{3}{4}$	Right sympathetic (in thorax) cut 3 hrs. after beginning; left sympathetic cut $\frac{3}{4}$ hr. later.
" 28	0.204	0.216	0.17	0.18	4	Both sympathetics cut in thorax, $2\frac{1}{2}$ hrs. after beginning.
" 29	0.120	0.122	0.18	0.18	$2\frac{1}{2}$	Both sympathetics cut 1 hr. after beginning.
" 30	0.221	0.228	0.13	0.14	6	Both sympathetics cut 5 hrs. after beginning. 1 hr. later, adrenals massaged.
" 31	0.242	0.234	0.18	0.16	$4\frac{1}{2}$	Both sympathetics cut 3 hrs. after beginning.
" 32	0.185	0.200	0.11	0.09	$5\frac{1}{2}$	Both major splanchnics cut $4\frac{1}{2}$ hrs. after beginning.
Dog 16	0.524	0.560	0.41	0.37	$6\frac{1}{4}$	Right sympathetic (in thorax) cut $4\frac{1}{2}$ hrs. after beginning, left sympathetic $1\frac{1}{4}$ hrs. later.

TABLE XIII.

Epinephrin Content in Cats Used for Experiments under Urethane and Ether without Section of Nerves to Adrenals.

No. of animal.	Weight of adrenal.		Epinephrin.		Duration of experiment.
	Left.	Right.	Left.	Right.	
	gm.	*gm.*	*mg.*	*mg.*	*hrs.*
33	0.178	0.164	0.14	0.14	$5\frac{1}{4}$
34	0.294	0.290	0.17	0.16	4
35	0.226	0.224	0.20	0.19	$3\frac{3}{4}$
36	0.256	0.238	0.20	0.19	$4\frac{3}{4}$
37	0.330	0.364	0.18	0.20	$5\frac{3}{4}$

Depletion of Epinephrin Store of the Innervated as Compared with That of the Denervated Adrenal in Animals, Dead of Infections, Etc.

The opportunity of making these observations was afforded by the death, from various causes, of a certain number of animals whose adrenal nerve supply had been cut on one side. The results are given in Table XIV. Complete or almost complete depletion of the epinephrin store of the still innervated gland was far more frequently observed in this group than in any of the experimental groups.

Infections of various kinds (pyogenic in Cats 38 and 41, pneumonia in Dogs 17 and 18) caused particularly marked depletion. It has been stated by a number of observers that the adrenals in animals killed by various infections are poor in epinephrin. Elliott[2] found a small epinephrin load in persons dead of various septic fevers. The results cited in Table XIV show clearly that this depletion is brought about, in great part at any rate, through the nerves supplying the adrenals. It is not possible to say from these results whether, in addition, there is any direct action of the poisons on the glands.

In a cat (No. 43) dead of distemper the epinephrin content was quite low (0.09 mg.), but in another cat (No. 42), dead of the same disease, the load was fairly high (0.28 mg.), although perhaps not as much as would have been expected in a healthy animal of the same size. The cat was exceptionally large, as were also the adrenals.

TABLE XIV.

Depletion of the Epinephrin Store of the Innervated Adrenal in Animals Dead from Infections, Etc.

No. of animal.	Weight of adrenal.		Epinephrin.		Remarks.
	Left.	Right.	Left.	Right.	
	gm.	*gm.*	*mg.*	*mg.*	
Cat 38	0.184	0.191	0.16	Trace.	Connections of left semilunar ganglion cut 8 days before death from infection (peritonitis).
" 39	0.312	0.304	0.09	"	Connections of left semilunar ganglion cut 5 days before death from unknown cause. Cat emaciated and in poor condition before operation.
" 40	0.170	0.198	Trace.	0.13	Connections of right semilunar ganglion cut 2 days before death from unknown cause. Cat in same condition as No. 39.
" 41	0.308	0.300	0.08	0.14	Connections of right semilunar cut 25 days before death. General infection; abscess in wound in neck and in flank; eyes infected.
" 42	0.396	0.402	0.28	0.28	Died of distemper 14 days after excision of left superior cervical ganglion.
" 43	0.278	0.272	0.09	0.09	Died of distemper 6 days after excision of left superior cervical ganglion.
Dog 17	0.530	0.570	0.50	0.31	Left major splanchnic cut 13 days before death from pneumonia.
" 18	0.360	0.350	0.30	0.08	Left major and minor splanchnics, 2 lumbar ganglia, and connections of celiac ganglion cut 14 days before death from pneumonia.
Rabbit 50	0.200	0.210	0.09	Trace.	Left adrenal excised 3 days before death from unknown cause.

The Effect of Electrical Stimulation of the Splanchnic Nerves on the Epinephrin Store.

Elliott has stated that stimulation of the splanchnics by induction shocks for periods varying from 3 to 7 hours produces only a slight effect on the epinephrin content. Stimulation was applied for a few minutes at a time followed by a few minutes of rest. "When the nerve was faradized continuously for a period of 2 hours the loss was almost inappreciable."

Our observations confirm those of Elliott, in as far as they show that it is much more difficult to demonstrate depletion by splanchnic

stimulation than under the influence of ether, operations, etc., despite the fact that more epinephrin is liberated in a given time during appropriate stimulation of the nerves than is liberated under anesthetics without stimulation. The difficulty of showing depletion with splanchnic stimulation is, therefore, not due to the small amount liberated. We cannot see any other explanation than that splanchnic stimulation also increases the rate of accumulation of the epinephrin in the gland.

Continuous stimulation of the splanchnic is not suitable for producing depletion, nor is stimulation for several minutes at a time with intervals of rest. For we have seen in experiments on liberation of epinephrin into the blood that the amount excreted rapidly declines when stimulation is kept up.

However, by stimulating only for a few seconds at a time, with intervals of 1 or 2 minutes' rest, and continuing the experiment over many hours we have been able to demonstrate distinct depletion of the gland whose nerve was excited as compared with the other. The reactions of the denervated eye were used to control the efficiency of the stimulation. A strength and duration of stimulation was chosen which gave good but not maximal dilatation of the pupil, and the rest intervals were increased or diminished from time to time, so as to keep each stimulation effective.

In Table XV is given a condensed protocol of an experiment on a cat in which over 300 successful stimulations of the right sympathetic trunk (with the splanchnic) in the thorax were made. The amount of epinephrin liberated by each stimulation was estimated by determining, at different times throughout the experiment, the amount of adrenalin which had to be injected to give approximately a pupil dilatation of the same magnitude, and beginning at the same time interval, as the dilatation caused by the liberated epinephrin. Both sympathetics were divided simultaneously in the thorax, so that the glands were under equal conditions, except for stimulation of the nerves of the right. At the end of the experiment the left adrenal weighed 0.159 gm. and contained 0.20 mg. of epinephrin, and the right adrenal weighed 0.181 gm. and contained 0.08 mg. of epinephrin.

The assays by adrenalin solution of the amount of epinephrin liberated from the right adrenal into the blood yielded the following results.

Epinephrin liberated by 316 stimulations, 0.31 mg.

Time.	No. of stimulations.	Adrenalin corresponding to each stimulation.	Total epinephrin.
		mg.	*mg.*
3.10–5.01 p.m.	47	0.002	0.09
5.04–7.28 p.m.	95	0.0013	0.12
7.32 p.m.–12.15 a.m.	174	0.0006	0.10

With the short time of each stimulation and the relatively long intervals of rest, it is to be supposed that the power of the adrenal to respond to stimulation of its nerves was conserved as much as possible. Nevertheless, the diminution in the response, which was not apparent for a long time during the earlier part of the experiment became more marked as time went on. This is strikingly illustrated in the table showing the amount of liberated epinephrin. The 174 stimulations in the last period yielded only 0.10 mg. of epinephrin, while forty-seven stimulations of the same duration (5 seconds) yielded in the first period of the experiment 0.09 mg. Add to this the fact that the strength of stimulation had to be considerably increased in the latter part of the experiment, as shown by the diminished distance between the coils given in the protocol.

The load of the right adrenal at the time the nerves were cut may be taken at 0.20 mg., the same as that of the left. This represents a good load for these small adrenals (the cat weighed only a little over 2 kilos), especially as some loss must have occurred in the hour after the administration of urethane before the nerves were divided. The right adrenal gave off to the blood 0.31 mg. of epinephrin, of which 0.12 mg. could have come from the store. This is on the assumption that no epinephrin whatever is liberated in the absence of innervation.[5] An amount approximately equal to the initial load must have been formed in the time the experiment lasted.

In other experiments lasting for a shorter time we have not been able to obtain evidence of depletion of the store by splanchnic stimulation. In one with over fifty stimulations, each of which caused the liberation of enough epinephrin to elicit good eye reactions, no deficiency in the store of the stimulated gland was found, although in the 4 hours of the experiment an amount of epinephrin not less than the maximal load of a single gland in the cat had been given off by it to the blood.

It is possible that in prolonged experiments the capacity of the adrenal to form epinephrin at an increased rate in response to splanchnic stimulation is lessened, and that this is the reason why depletion of the store can then be demonstrated.

TABLE XV.

Condensed Protocol. Cat 44, Weight 2.25 Kilos. Superior Cervical Ganglion Excised 17 Days before. Animal Pregnant. Cannulas in Right Femoral Vein and Trachea. Unless Otherwise Mentioned, Time of Stimulation Was Always 5 Seconds. The Left Sympathetic Was Stimulated Only Once.

Time.		Interval between beginning of stimulation and of pupil dilatation.
p. m.		*sec.*
1.40	Urethane, 3 gm. by stomach tube	
2.20	A little ether. No more ether during experiment	
2.45	Both sympathetics (with splanchnic) ligated and cut in thorax	
3.10	Stimulated left sympathetic (11 cm. between coils)	9.6
3.30–4.08	Stimulated right sympathetic (3–5 sec.) (9–10 cm.) 10 times	6.6–7.8*
4.08	Adrenalin injections. Same pupil reactions given by 0.3 cc. (1 : 140,000)†	
4.09–4.27	Stimulated right sympathetic (9 cm.) 15 times	6.0–7.2
4.27	Adrenalin injections. Reaction, 0.3 cc.	
4.28–4.36	Stimulated right sympathetic (9 cm.) 6 times	7.4–8.8
4.36	Adrenalin injections. Reaction, 0.3 cc.	
4.40–4.46	Stimulated right sympathetic (9 cm.) 5 times	7.6–8.2
4.47	Adrenalin injections. Reaction, 0.3 cc.	
4.49–5.01	Stimulated right sympathetic (9 cm.) 11 times	6.8–7.8
5.02	Adrenalin injections. Reaction, 0.3 cc.	
5.04–5.21	Stimulated right sympathetic (9 cm.) 14 times	7.2–8.0
5.22	Adrenalin injections. Reaction, 0.25 cc.	

* The time interval of the pupil reaction was constant throughout the experiment. Only the minimum and maximum times are given in this column for the various series of stimulations. The times for the pupil reactions yielded by these 10 stimulations may be given as an example (7.4, 7.0, 7.4, 7.8, 7.8, 7.2, 7.2, 7.6, 6.6, 7.2). The number in parenthesis after stimulation gives the distance between the coils in cm.

† Only the quantities of adrenalin found in the trial injections to correspond with the effects of the splanchnic stimulations are reproduced from the protocol. The adrenalin solution was itself assayed. The corrected concentration is what is given in the protocol. A freshly made solution in 0.9 per cent sodium chloride was used for each assay. The adrenalin solution injected was always a 1:140,000. The concentration of the solution is therefore henceforth omitted in the protocol.

TABLE XV—*Concluded.*

Time.		Interval between beginning of stimulation and of pupil dilatation.
p. m.		*sec.*
5.25–5.36	Stimulated right sympathetic (9 cm.) 8 times	7.2–8.2
5.38	Adrenalin injections. Reaction, 0.2 cc.	
5.40–5.59	Stimulated right sympathetic (9 cm.) 15 times	6.6–8.6
6.00	Adrenalin injections. Reaction, 0.2 cc.	
6.02–6.18	Stimulated right sympathetic (9 cm.) 13 times	6.8–7.4
6.20	Adrenalin injections. Reaction, 0.2 cc.	
6.22–6.36	Stimulated right sympathetic (9 cm.) 12 times	6.4–7.2
6.40	Adrenalin injections. Reaction, 0.2 cc.	
6.42–6.53	Stimulated right sympathetic (9 cm.) 7 times	7.0–7.4
6.58	Adrenalin injections. Reaction, 0.2 cc.	
7.00–7.28	Stimulated right sympathetic (9 cm.) 26 times	6.6–7.2
7.31	Adrenalin injections. Reaction, 0.2 cc.	
7.32–7.50	Stimulated right sympathetic (9 cm.) 18 times	6.2–7.2
7.54	Adrenalin injections. Reaction, 0.1 cc.	
7.55–8.10	Stimulated right sympathetic (8 cm.) 13 times	6.2–8.8
8.12	Adrenalin injections. Reaction, 0.1 cc.	
8.15–8.38	Stimulated right sympathetic (8 cm.) 20 times	6.4–7.8
8.40	Adrenalin injections. Reaction, 0.1 cc.	
8.42–9.20	Stimulated right sympathetic (7.5 cm.) 26 times	6.4–7.8
9.22	Adrenalin injections. Reaction, 0.1 cc.	
9.26–9.48	Stimulated right sympathetic (7.5 cm.) 14 times	6.8–8.0
9.50	Adrenalin injections. Reaction, 0.1 cc.	
9.53–10.25	Stimulated right sympathetic (7.5 cm.) 24 times	6.8–7.8
10.27	Adrenalin injections. Reaction, 0.1 cc.	
10.30–10.50	Stimulated right sympathetic (7.5 cm.) 16 times	6.4–7.8
10.53	Adrenalin injections. Reaction, 0.1 cc.	
10.55–11.30	Stimulated right sympathetic (7.5 cm.) 23 times	6.8–8.8
11.34	Adrenalin injections. Reaction, 0.1 cc.	
11.37 p.m.–12.15 a.m.	Stimulated right sympathetic (7.5 cm.) 20 times	6.8–9.0
12.15 a. m.	Adrenalin injections. Reaction, 0.1 cc.	

Circulation good up to the end of the experiment; the heart was beating well, although the pulse was gradually becoming more rapid toward the last hour of the experiment.

The left adrenal weighed 0.159 gm. and contained 0.20 mg. of epinephrin.

The right adrenal weighed 0.181 gm. and contained 0.08 mg. of epinephrin.

SUMMARY.

1. No evidence has been obtained that in cats and dogs with the nerves of one adrenal cut, emotional disturbances cause depletion of the epinephrin store of the normally innervated adrenal as compared with its fellow.

2. The depletion of the epinephrin store in cats under morphine is not dependent upon so called morphine fright, since a similar depletion is found in dogs in which, as is known, morphine produces symptoms the reverse of those of fright.

3. The signs of morphine fright can all be elicited by administering morphine to a cat in which one adrenal has been removed and the nerve supply of the other cut, and in which accordingly no detectable liberation of epinephrin takes place.

4. The reactions of the denervated iris elicited by emotional disturbance, asphyxia, or etherization in a cat, one of whose adrenals has been removed and the nerves of the other cut, do not differ from these reactions in cats whose adrenals have not been interfered with.

5. The influence of postoperative edema of the adrenal in diminishing the epinephrin load, and the recuperation of the load after a time, have been studied in rabbits.

6. The diminution in the epinephrin store of the adrenals which follows operations on animals (postoperative depletion) has been studied. It is only in part associated with the anesthesia, since it may be as marked 6 or 8 hours after an operation lasting less than 1 hour as after 6 or 8 hours' anesthesia without operation.

7. One adrenal was removed in rabbits and the epinephrin content of the remaining gland assayed at varying periods of time after removal of the first, the periods being longer than the time necessary for recovery from the postoperative depletion. In general, the second adrenal contained more epinephrin than the first, sometimes double the amount.

8. Marked depletion of the epinephrin store of innervated adrenals as compared with the corresponding denervated glands was seen in animals dead of infections of various kinds.

9. As shown by Elliott, diminution of the stock of epinephrin in the adrenal through electrical stimulation of the splanchnics is not easy to demonstrate, despite the fact that the liberation of epinephrin into the blood is notably increased by the stimulation. With short periods of stimulation, however, repeated over a long time at intervals just long enough to prevent fatigue, it has proved possible to demonstrate a distinct depletion.

A STUDY OF EXPERIMENTAL NON-HEMOLYTIC STREPTOCOCCUS LESIONS IN VITALLY STAINED RABBITS.

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PLATES 49 TO 51.

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Within the last 15 years much has been written about the streptococcus in rheumatic fever, and emphasis has been laid on the bacteriological rather than on the pathological aspects of the disease. The reasons for this were twofold: (1) the low mortality in rheumatic fever made the material for pathological study scanty; (2) the frequently reported occurrence of streptococci in the various lesions of rheumatic fever cases stimulated so much interest in the bacteriology of the disease that other phases of the problem have been neglected.

HISTORICAL.

The most important recent contribution to the pathology of rheumatic fever is that of Aschoff (1), who in 1904 described certain small collections of cells in the myocardium which he considered characteristic of rheumatic fever. Aschoff spoke of them as "submiliary nodules." They are usually found in the neighborhood of the smaller arterioles and consist of a collection of unusually large cells with one or more large irregularly shaped nuclei. These giant cells, according to Aschoff, are often arranged like a rosette about a center of necrotic confluent cellular protoplasm. Just outside the giant cells, which resemble somewhat those seen in Hodgkin's disease, there is a peripheral zone of leukocytes, in which lymphocytes predominate. The peripheral cells form streaks or out-shoots into the connective tissue septa. In these streaks are also found the adventitia or wandering cells which appear in all inflammatory processes. They arise from the vessels and it is from them, Aschoff thinks, that the giant cells develop.

In the following year Geipel (2) studied five cases of rheumatic myocarditis. His description of the submiliary nodule agrees with Aschoff's, but he disagrees with Aschoff as to the origin of the giant cells, for he believes that they arise from connective tissue. According to Geipel, the connective tissue cells swell

and in some instances fuse together to form the multinucleated giant cells. Both Aschoff and Geipel describe the gradual conversion of the submiliary nodule into a patch of fibrous tissue. Aschoff claims that these foci are specific for rheumatic fever, but Geipel will not admit this.

Fraenkel (3) confirmed Aschoff's observations as to the specificity of the nodules, but does not commit himself as to the origin of the giant cells. Coombs (4) has devoted considerable study to Aschoff's bodies, and, like Geipel, considers them "the product of a connective-tissue proliferation in response to an irritant."

Bracht and Wächter (5) found the Aschoff bodies in three out of four cases of rheumatic myocarditis. They also conclude that the giant cells are derived from the fixed connective tissue cells, basing their opinion on the fact that these cells stain with the Unna-Pappenheim stain like swollen fibroblasts. These investigators were the first to produce experimental focal lesions in the myocardium of rabbits by injecting them with streptococcus. Rabbits injected with *Streptococcus viridans* showed considerable necrosis of the cardiac muscle fibers. The older foci showed a considerable number of lymphocytes and fibroblasts and a few giant cells. In still older lesions, fibrous scars were forming. In animals, on the other hand, that were injected with *Streptococcus hemolyticus*, the heart muscle showed small abscesses and dense infiltration of polynuclear leukocytes.

Takayasa (6) describes the heart changes in one case of rheumatism in which he found Aschoff bodies, but he offers no theory as to the origin of the giant cells.

Thalhimer and Rothschild (7) have recently studied the cardiac changes in rheumatic fever and chorea and conclude that the presence of Aschoff nodules signifies a previous rheumatic infection.

Jackson (8) injected hemolytic streptococci and also a *Streptococcus viridans* into rabbits. With the latter she produced foci consisting of degenerated muscle fibers around which are large mononuclear cells, large multinucleated giant cells, and proliferating connective tissue. In other lesions "the cells occupying the center of the lesion are very large with round or oval lightly stained nuclei and a large amount of granular cytoplasm in which bacteria are often seen." About these cells are many smaller, more deeply stained cells which are irregularly shaped and definitely outlined, with round deeply stained eccentric nuclei (plasma cells). In the relative absence of necrosis and of polynuclear leukocytes and in the abundance of large endothelial cells, these lesions are peculiar, showing the same characteristics emphasized by Coombs and others as characteristic of the focal human lesions in rheumatism.

De Vecchi (9) in 1912 injected the blood from cases of rheumatic fever into rabbits and was able thereby to produce lesions in the heart muscle. The lesions consisted of diffuse infiltrations of cells usually about a small vessel. The muscle fibers are often devoid of contractile tissue and appear homogeneous or in amorphous blocks. The cellular foci are composed of (1) leukocytes, mostly lymphocytes, (2) cells with large vesicular nuclei containing a network of chromatin and

scanty protoplasms. De Vecchi remarks that these experimental lesions resemble the rheumatic nodules, but his description and drawings do not support this claim. They might more readily be explained as a result of the injection of a foreign protein, as shown by Longcope (10).

Coombs, Miller, and Kettle (11) inoculated rabbits with streptococcus and produced cardiac lesions which differed in no essential manner from the lesions of human rheumatism.

Schloss and Foster's (12) experiments on monkeys were performed with *Streptococcus hemolyticus;* hence they are hardly comparable with the experiments which have been described above. They found the myocardial vessels thickened and surrounded in places by groups of cells which took a deep red color with pyronin methyl green. These cells looked like plasma cells, but were a little larger. Their nuclei were eccentric and stained light blue or green. Thalhimer and Rothschild (7) injected rabbits with *Streptococcus mitis* (*viridans*) and produced focalized myocardial lesions which they describe as follows:

"The lesions are entirely different from the submiliary myocardial rheumatic nodules described by Aschoff. . . . . The lesions in the myocardium of the rabbit are a combination of degenerative and productive processes. Degeneration of the muscle fibres occurs either accompanied by or soon followed by a proliferation of the interstitial tissue which is disproportionate to the extent of the myocardial change."

The following facts are evident from this review of the literature. (1) Opinion is almost unanimous in considering Aschoff bodies as specific for rheumatic fever. (2) There is considerable diversity of opinion as to the origin and character of the large cells which for the most part constitute the lesion. By Coombs they are considered endothelial; by Geipel, Bracht and Wächter, and others, fibroblasts; while Aschoff himself looks upon them as transformed macrophages or wandering cells. (3) Efforts to reproduce Aschoff bodies experimentally have met with varying results in the hands of different investigators. Coombs and de Vecchi and Jackson all claim to have produced lesions which they consider essentially identical with Aschoff bodies. Bracht and Wächter, and Thalhimer and Rothschild produced lesions which are not identical with Aschoff bodies and are easily distinguished from them.

It is obvious that once granted that Aschoff bodies are a specific manifestation of rheumatic fever, the experimental production of them by injection of streptococci would be strong evidence in favor of the streptococcal origin of rheumatism. The present study was undertaken to determine, if possible, the origin of the peculiar cells

in the myocardial lesion; as the work progressed it seemed desirable to include also a study of the other structures involved, especially the joints.

In view of the disagreement as to the origin of the cells which constitute the Aschoff body in the human heart and the lack of harmony in the results from experimental study of the lesion, it was thought that the employment of a vital stain might prove of value.

Evans, Bowman, and Winternitz (13) have recently used this method with considerable success for the histological study of the miliary tubercle, and by vital stains demonstrated that in the liver both the epithelioid and giant cells of the tubercle develop from the endothelial lining of the capillaries.

In using the vital stain I have followed the method of Goldmann (14) who first reported his experiments with trypan blue and isamin blue in 1912. Goldmann employed the dyes in 1 per cent aqueous solutions and administered them either subcutaneously, intraperitoneally, or intravenously. In any case, the animals rapidly took on a deep blue color and at autopsy the various organs and tissues showed more or less pigmentation with the dye. Microscopically it was at once obvious that certain cells had taken up the blue granules, while others had not. For instance, Goldmann noted that the white corpuscles of the blood and the plasma cells never took the blue; on the other hand, the so called wandering cells, which are present throughout all the tissues, invariably showed phagocytosis of the granules. These wandering cells have received a variety of names, being variously known as macrophages, polyblasts, adventitia cells, clasmatocytes, or endothelioid cells, and Goldmann lengthened the list by calling them pyrrhol cells. He found them in the subcutaneous tissue and in all the internal organs and very abundant in the pregnant uterus and in healing wounds. The characteristic feature of these cells is their active phagocytosis of bacteria and foreign matter of any kind. Goldmann thinks that they eventually stretch to form the spindle cells of young connective tissue, thereby losing their affinity for vital and fat stains as well as their phagocytic power.

Evans (15) has recently described these vitally staining cells in detail. He speaks of them as macrophages and classifies them as follows: (1) endothelial macrophages which line the capillaries in the liver, spleen, bone marrow, and hemal glands, and the lymphatic sinuses of the lymph glands; (2) macrophages which are more or less fixed, such as the cells of the reticulum of lymph glands, spleen, and bone marrow; also resting wandering cells in the connective tissues, especially numerous in the omentum; (3) the free macrophages which comprise the large mononuclear cells of the serous cavities and those present in the lymphatic sinuses of lymph glands and sometimes seen in the hepatic and splenic capillaries. Evans has demonstrated that the intramuscular macrophages may develop from endothelium, but he is not willing to go so far as Mallory, who considers all macrophages of endothelial origin.

It will be seen from this review that the vital stain is of considerable value in the study of cellular foci as helping to distinguish the various types, leukocyte, endothelium, fibroblast, and macrophage from one another.

Methods.

In the present study, the technique employed was as follows: Freshly distilled water was heated to 56°C. and enough trypan blue added to make a 1 per cent solution. While still warm the dye was injected into the rabbits usually by the intravenous route, though occasionally subcutaneous or intraperitoneal injections were substituted. The dye has a tendency to produce thrombosis of the ear veins. The rabbits were usually injected first with streptococci, but in some cases the trypan blue was administered previously. Histologically it seemed to make no difference which preceded the other. The usual dose of trypan blue was 10 cc. of the 1 per cent solution every day for 1 to 2 weeks. The number of trypan blue injections varied from one to twenty-three, the average number being twelve. By this method it was found that the tissues of the rabbits acquired a deep blue color and at the same time the animals suffered little physical deterioration. Most of the rabbits were killed by intracardiac injection of formalin. The rabbit was anesthetized, and the jugular and femoral veins were exposed. The sternum was then rapidly removed and a cannula inserted into the left ventricle. A 4 per cent solution of formalin was then allowed to run in through the cannula and at the same time the jugular and femoral veins were severed. The animal was thus rapidly exsanguinated, and in a few minutes formalin could be detected in the venous outpourings. The perfusion with formalin was continued for 10 to 15 minutes, usually until several hundred cc. of the solution had been injected. A complete autopsy was then performed and thin pieces of tissue were preserved in 4 per cent formalin for sections. Sections were also taken from the infected joints and decalcified in Müller's fluid. Films and sometimes cultures were made from the pus in the infected joints. The intracardiac injection of formalin did not interfere with the recovery of streptococci in culture from the joints.

Thirty-eight rabbits in all were used in the experiments (Table I). In addition, three controls were injected with trypan blue but received

TABLE I.

No. of rabbit.	Weight.	No. of trypan blue injections.	No. of streptococcus injections.	Duration of life after 1st injection of streptococci.	Arthritis.	Lesions in heart.	Lesions in liver.	Lesions in kidneys.	Remarks.
	gm.			*days*					
1	1,820	2	1 (59–F)	1	0	Focal necrosis.	Focal necroses.		
2	1,670	2	1 (59–F)	2	0	" "	" "	Infiltration of leukocytes.	
3	1,900	2	1 (59–F)	7	0	Endocarditis, acute. Pericarditis, acute.	0	Parenchymatous changes.	
4	2,230	3	1 (59–F)	7	0	Focal necrosis and infiltration.	0	Infiltration of small round cells.	Streptococcus recovered from heart's blood at autopsy.
5	1,850	3	1 (59–F)	7	0	Focal necrosis.	0	Severe parenchymatous nephritis, focal necroses.	"
6	1,715	9	4 (A–30)	26	0	0	Infiltration of leukocytes in lobules.		
7	1,620	0	3 (A–30)	18	Acute. Left wrist.	Acute pericarditis.	Infiltration of lymphoid cells.	Infiltration of lymphoid cells.	Died of pneumonia.
8	1,720	7	4 (59–F)	26	Subacute. Left shoulder.	0	0	Collection of lymphoid cells in cortex.	

9	1,800	0	3 (59–F)	12	Acute. Both ankles, left wrist, right shoulder.	0	Diffuse infiltration of round cells.	Collection of lymphoid cells in cortex.	Died of pneumonia. Streptococcus from joints.
10	670	6	1 (A–30)	8	Acute. Right knee.	0	Marked cloudy swelling.	Collection of lymphoid cells.	
11	554	0	1 (A–30)	1	0	0	0	0	Culture from heart's blood sterile.
12	692	18	4 (59–F)	70	Subacute. Right elbow, left shoulder.	Focal necrosis and infiltration. Repair.	Focal necroses, early cirrhosis, endocarditis.	Collection of lymphoid cells.	
13	490	1	1 (59–F)	1	0	0	0	0	Died.
14	634	9	2 (A–49)	19	Acute. Both knees and shoulders.	0	0	Collection of lymphoid cells.	
15	624	6	2 (A–49)	17	Acute. Both shoulders, left wrist.	A few foci of round cell infiltration.	Focal necroses.	"	
16	635	1	1 (59–F)	1	0	Foci of round cell infiltration.	0	0	Died.
17	600	3	1 (59–F)	8					Incomplete autopsy.
18	425	5	1 (A–32)	6	0	Focal necrosis and round cell infiltration.	Small adenomata of ducts.	0	
19	779	6	1 (59–F)	19					Incomplete autopsy.
20	500	6	1 (A–32)	7	0	0	Coccidia.	0	

TABLE I—*Continued.*

No. of rabbit.	Weight.	No. of trypan blue injections.	No. of streptococcus injections.	Duration of life after 1st injection of streptococci.	Arthritis.	Lesions in heart.	Lesions in liver.	Lesions in kidneys.	Remarks.
	gm.			*days*					
21	682	2	1 (59–F)	1					Incomplete autopsy.
22	1,610	14	20 (59–F)	225	Chronic. Both shoulders, left wrist.	Foci of round cell infiltration.		Infiltration of lymphoid cells.	
23	1,480	14	13 (A–26)	180	Chronic. Both shoulders.	0		0	
24	1,800	14	17 (A–32)	180	Chronic. Right shoulder.	0		0	
25	1,895	4	8 (59–F)	40	Subacute. Both shoulders.	Acute endocarditis and pericarditis. Foci of round cell infiltration. Thrombosis of vessels.		0	
26	1,820	14	8 (59–F)	50	Subacute. Both shoulders.	A few foci of cellular infiltration.		Parenchymatous changes.	
27	1,880	14	8 (59–F)	140	0	0	Moderate cirrhosis.	0	
28	1,760	14	8 (59–F)	50	Subacute. Both shoulders.	Foci of necrosis and cellular infiltration.		0	
29	1,830	14	5 (59–F)	50	0	0		0	
30	2,050	9	2 (A–49)	14	0	0	Central necroses.	0	
31	2,490	18	3 (A–49)	28	Acute. Right elbow.	Foci of cellular infiltration. Repair.	Coccidia.	Collection of lymphoid cells.	Streptococcus recovered from joints.

32	2,200	8	1 (A-49)	10	Acute. Right elbow, left knee.	0	0	Slight sclerosis, infiltration of lymphoid cells.	Streptococcus recovered from joints.
33	2,295	18	3 (A-49)	28	Acute. Both elbows and knees, both wrists and left ankle.	0	0	Collection of lymphoid cells.	"
34	2,640	14	2 (A-49)	21	Acute. Right knee. Synovitis, right leg.	Foci of necrosis and cellular infiltration.	0	0	"
35	1,890	19	3 (A-49)	26	Acute. Left shoulder.	Foci of cellular infiltration.	0	Marked parenchymatous nephritis.	Rabbit received very large doses of streptococci (100–200 cc.) (broth culture).
36	1,700	17	2 (38-D)	12	Acute. Synovitis, both heels.	Large foci of necrosis, cellular infiltration, and repair.	0	Parenchymatous nephritis, infiltration of lymphoid cells.	Rabbit received very large doses of streptococci.
37	1,760	6	1 (38-D)	1	0	0	Central necroses.	0	"
38	1,810	6	1 (38-D)	1	0				
39	1.580	16	0		0	None. Many macrophages.	Many macrophages.	0	Control.
40	1,680	23	0		0	" " "	" "	0	"
41	1,770	23	0		0	" " "	" "	0	"

no streptococci. The rabbits were usually of medium size, varying in weight from 425 to 2,500 gm. For one series, an attempt was made to use five young rabbits, but their veins were not well adapted to the trypan blue injections.

Six different strains of streptococci were employed for the experiments. They were obtained from blood culture from the following sources:

1. Rheumatic fever (59–F).
2. Rheumatic fever (A–49).
3. Rheumatic fever (38–D).
4. Infectious endocarditis (subacute) (A–30).
5. Infectious endocarditis (subacute) (A–32).
6. Infectious endocarditis (subacute) (A–26).

The six strains all belonged to the non-hemolytic group of streptococci. Four were green producers, while two, A–26 and A–32, produced no change whatever in blood.

The number of streptococcus injections varied from one to four and were separated by 5 to 10 day intervals. The average number of injections was two. This excludes the eight immunized animals (Nos. 22–29) which first received several injections of killed streptococci and then a series of injections (five to twenty) of living streptococci.[1] The dosage of streptococcus culture used in my experiments has varied considerably. In the earlier work I used 5 to 20 cc. of a 24 hour broth culture. In the later experiments much larger doses were given (three to five rabbit blood agar slants), and in the last series 100 to 150 cc. of a broth culture were injected at one time.

RESULTS.

Joint Lesions.

In this investigation I have given considerable study to the pathology of the joints, for in reviewing the literature it was noticeable that very little attention has been paid to the pathological anatomy of arthritis, either in the human being or experimental animal. Poyn-

[1] The privilege of studying the lesions in the immunized rabbits was granted to me by Drs. Swift and Kinsella who were employing them for the study of another problem.

ton and Paine (16), Cole (17), and Jackson (8) have described the gross, and to some extent the microscopic appearance, but the cellular changes have not been carefully studied. In every case of arthritis in the present series, sections have been taken through the joints involved and studied microscopically. The sections were all decalcified in Müller's fluid, which did not injure the vital stain to any great extent. The sections were cut by the paraffin method and stained (1) with hematoxylin and eosin, (2) with aqueous cochineal (to bring out vitally stained cells), and (3) by the Gram-Weigert method for bacteria. The Unna-Pappenheim stain could not be used on account of the formalin fixation. By referring to the table it will be seen that of the thirty-eight rabbits injected with streptococci, nineteen, or 50 per cent developed arthritis; in seven the disease was monarticular, and in twelve polyarticular. The shoulder was the joint most frequently involved; the right nine times, the left ten. The other large joints were affected with about equal frequency. In many cases it was difficult to determine the exact duration of the arthritis, for the reason that the rabbit showed no symptoms. Many infected joints were not discovered until the autopsy was performed.

The rabbits with arthritis lived from 8 to 225 days after receiving their first injection of living streptococci. Four animals lived less than 2 weeks; eight of the nineteen rabbits lived from 2 to 4 weeks after their first injection. The remaining seven lived from 6 to 32 weeks.

The four rabbits that lived or were killed in less than 2 weeks after receiving streptococci (Nos. 9, 10, 32, and 36) had arthritis in a very acute form. Two of these rabbits received only one injection each of streptococci; another received two, and the last three injections. Faber (18) found that it usually took more than one injection of streptococci to produce arthritis. As a rule, this is true, but not invariably, as our results show. In the gross these joints were moderately swollen and contained bluish viscid mucoid material, films of which showed many pus cells filled with coarse blue granules of trypan blue. In two of the cases streptococci were recovered in cultures from the joint exudate. Microscopical sections through the infected joints show extensive infiltration of the synovial membrane with leukocytes, chiefly of the polynuclear type. In addition there

are present a few large endothelioid cells (macrophages) which contain blue granules and are similar in every respect to the large cells found in the joint exudate. The membrane is thickened from edema and cellular infiltration. The synovial membrane is covered with an exudate of leukocytes and a few endothelioid cells. The cartilage is intact and shows no infiltration. The marrow shows a slight hyperplasia. The capsule is little if any changed.

In the second group of cases that lived for 2 to 4 weeks after the first injection of streptococci (Nos. 7, 8, 14, 15, 31, 33, 34, and 35), the gross appearance of the infected joints differed in no way from those of the first group.

Microscopically, however, there are some differences. In these rabbits the infection is obviously of longer duration. In some places the cartilage is superficially necrotic and the destroyed portion is being replaced by connective tissue. The bone marrow shows considerable hyperplasia. The synovial membrane is thickened and densely infiltrated with leukocytes and the same large endothelioid cells filled with blue granules (Fig. 1). Lymphoid and plasma cells are more numerous in the infiltration and exudate than in the first group.

There are signs of beginning organization in the exudate and it is interesting in this connection to study the part of the large endothelioid or macrophage cells. On the surface they appear as large round, or oval cells packed with coarse blue granules. Deeper down in the exudate near the synovial membrane these cells have apparently become pulled out into fusiform and stellate shapes, appear smaller, and contain fewer and finer granules. The impression is obtained that the macrophages, having accomplished their first duty as phagocytic cells, have become gradually transformed after this function is completed into fibroblasts to assist in the process of repair. Eventually they become adult connective tissue cells and lose their phagocytic power entirely.

In the third group of cases (Nos. 12, 22, 23, 24, 25, 26, and 28) many of the infected joints began to show chronic changes, such as great thickening of the capsule with considerable limitation of motion; the bone was very friable and crumbled easily when sections were sawed for microscopical examination. There was no bony ankylosis, however, in any of the cases.

Microscopical sections through these joints show extensive necrosis of the cartilage which sometimes extends down into the bone. There is marked hyperplasia of the marrow which has replaced the cancellous bone to a great extent. The synovial membrane is thickened and the deeper part composed of newly formed connective tissue; the superficial part is very cellular. The cells are leukocytes, mostly lymphoid and plasma cells, though there are a moderate number of polynuclear cells also present and large numbers of macrophages which appear to be undergoing the transition into fibroblasts, as just described. No bony changes were observed, even in the more chronic cases, but there were hyperplasia of the bone marrow and occasional thrombi or foci of granulation tissue in the marrow.

It will be seen from these descriptions that the changes which take place in experimental arthritis represent a gradual transition from a stage of acute inflammation in which there is little more than an infiltration of leukocytes into the synovial membrane and an outpouring of mucoid material, leukocytes, and macrophages into the synovial fluids to a stage of subacute or chronic inflammation in which the following changes occur. The synovial membrane and the cartilage undergo more or less necrosis. The cellular exudate in the synovial membrane consists chiefly of lymphoid cells and macrophages. There is marked hyperplasia of the bone marrow with more or less absorption of the cancellous bone. It is possible that if some of these rabbits had lived long enough, new formation of bone and possibly ankylosis would have occurred, as described by Poynton and Paine. The capsule of the joint thickens as a result of the long continued inflammation. The changes resemble those observed in cases of acute infectious arthritis in man which become subacute and finally pass into the chronic stage of arthritis. From the standpoint of the pathology and histology of experimental arthritis, there is little to differentiate it from human arthritis of the infectious or metastatic type. It has been observed by previous investigators of experimental streptococcus arthritis that complete recovery of the joint may take place as in human rheumatism. Unfortunately, I have not had an opportunity to study any joints of this type.

Cardiac Lesions.

The hearts were studied in considerable detail and it was chiefly in the hope of throwing some light on the foci found in the heart in experimental streptococcus arthritis that the rabbits were vitally stained. Almost all the sections studied were from the left ventricle, and particular care was taken to include the papillary muscles in the sections.

Of the thirty-eight rabbits injected with the streptococci, eighteen showed pathological changes in the heart or pericardium; eleven of the eighteen belonged also to the arthritis series. In three rabbits complete autopsies were not performed. Rabbit 3 showed an endocarditis and pericarditis. In Rabbit 25 a vegetative endocarditis was found associated with pleurisy and pneumonia. Rabbit 7 had a pericarditis. In the remaining fifteen the lesions were in the myocardium. These lesions were usually not visible with the naked eye on account of the trypan blue stain. The earliest focal lesions observed have been small areas of necrosis in the muscle fibrillæ (Fig. 2). At first these consist only of necrotic fragments of muscle with here and there a surviving nucleus. At this stage no infiltration of leukocytes or wandering cells has occurred. These foci are usually small, but occasionally one sees necrotic areas of considerable extent situated in the muscle, never in the supporting stroma, and sharply defined from the surrounding healthy parenchyma. The line of demarcation from healthy tissue is emphasized by the vital staining of the necrotic foci, which take a light blue color due to the deposition in them of very fine blue granules of trypan blue. A later stage of such focal necroses is characterized by the presence of leukocytes, usually of the lymphoid type, and macrophages filled with blue granules (Fig. 3). Focal necroses were observed in nine cases. In three of these (Nos. 1, 2, and 5) the focal necroses were the only lesions present. The other six (Nos. 4, 12, 18, 28, 34, and 36) showed in addition to necrosis more or less extensive cellular infiltration. In seven other cases, foci of infiltration, chiefly by lymphoid cells, were present with little or no necrosis (Fig. 4).

The foci of cellular infiltration were of particular interest, especially as regards their resemblance to Aschoff's bodies. The foci

with necrotic centers surrounded by leukocytes and macrophages (Fig. 3) bore the nearest resemblance to the submiliary nodules of rheumatic fever, but even in these the resemblance was not close. In the first place, the location of the lesions in the rabbit heart is different from that in the human heart. In the rabbit I have always found the necrotic foci in the heart muscle proper and not related in any way to the blood vessels. Sometimes smaller collections of lymphoid and macrophage cells are found in the stroma, but this is the exception rather than the rule. The submiliary nodule is always found in the supporting connective tissue, usually about a blood vessel. In the rabbit's heart the necrosis is definite; in the human heart it is slight and of the hyaline type. There is a difference, too, in the cellular exudate about the necrotic foci. In the rabbit the cells are chiefly lymphoid and plasma cells and large macrophages. In the human lesion there are, in addition to these types, a considerable number of giant cells which resemble those seen in Hodgkin's disease. Furthermore, the radial arrangement of the cells in a submiliary nodule is characteristic, whereas in the experimental nodule the cells are arranged in a purely haphazard manner.

In Rabbit 36 I succeeded in demonstrating streptococci in one of the myocardial lesions. This rabbit, however, had received such large doses of streptococci that perhaps too much significance should not be attached to this point. Thalhimer and Rothschild were unable to demonstrate bacteria in the myocardial foci which they studied, and I have not found them in any other of my own cases.

In a number of cases, especially Nos. 12, 31, and 36, there were signs of repair (Fig. 5), and here, as in the joints, the macrophages seemed to play an important part. They were present in large numbers and were apparently being converted into fibroblasts.

It may be said, then, that while the myocardial lesions in experimental streptococcus arthritis follow in a general way the course of those occurring in the rheumatic human heart,—that is, necrosis and infiltration and finally proliferation with the formation of scars,—the histological appearance of the two lesions when carefully studied, is different.

Endocarditis and Pericarditis.

Sections through the vegetations on the heart valves in Rabbits 3 and 25 showed a fibrinous network, partially organized, and infiltrated with leukocytes and a moderate number of macrophages. Sections through the pericardial exudates presented similar pictures (Rabbits 3, 7, and 25).

Lesions in the Kidneys.

In the study of the rabbit's kidneys the investigator is always handicapped by the frequency of pathological changes in supposedly normal kidneys. Christian, Smith, and Walker (19), Ophüls (20), Longcope (21) and others have found round cell infiltration and patches of sclerosis in 22 to 33 per cent of kidneys of normal rabbits.

In the present series of thirty-seven cases in which the kidneys were examined microscopically, the kidneys of the three controls, from rabbits that received trypan blue but no streptococci, were normal; and of the remaining thirty-four from rabbits that had received injections of streptococci, eighteen, or 52 per cent, showed definite pathological changes. Of these, fourteen were associated with arthritis. As in all there were nineteen cases of arthritis, it follows that fourteen out of the nineteen, or 74 per cent, were associated with renal changes. In nine cases the lesion consisted of collections of lymphoid cells in the cortex with more or less patchy sclerosis. In four cases there was a well developed parenchymatous nephritis, with extensive destruction of the tubular epithelium of the convoluted tubules. In one case both parenchymatous and interstitial changes were noted (No. 36).

The glomerular changes in streptococcus infections have been studied by Baehr (22), Löhlein (23), and others. Baehr has described characteristic glomerular lesions in infectious endocarditis due to the lodgment of bacterial emboli in the glomerular capillaries.

Lesions in the Liver.

In six cases the liver showed focal necroses, which were usually about the central veins and were themselves in most cases surrounded by a zone of leukocytes. In only two of the six cases was

there an associated arthritis. In two other cases there was a well marked cirrhosis (Nos. 12 and 27). A slight degree of round cell infiltration and cirrhosis was present in some of the other cases, but the change was not sufficiently marked to be significant.

Lesions in Other Organs.

The spleen and lymph glands were enlarged in all the rabbits. Microscopical sections showed hyperplasia of the lymphoid tissue and large numbers of macrophages. These changes were apparently due to the vital stain as they were also observed in the control rabbits. In Rabbit 9 there were infarcts in the spleen.

The lungs showed nothing of interest except in two cases. Rabbits 7 and 9 both died of pneumonia. Microscopical sections showed the usual exudate in the pulmonary alveoli.

DISCUSSION.

The injection of one or more doses of *Streptococcus viridans* into the vein of a rabbit produces in many cases an acute arthritis which bears some resemblance to the arthritis of rheumatic fever, but is perhaps more nearly analogous to the so called acute or subacute infectious arthritis so frequently associated with focal infections in the teeth, tonsils, or other parts of the body. Microscopically, joint sections from vitally stained animals show in addition to numerous leukocytes, a considerable number of large endothelioid cells or macrophages, which take the vital stain. These cells migrate from the tissue into the joint exudate and could not be seen to develop from the endothelial lining of the joint as commonly supposed. As the infection becomes older these macrophages become more and more numerous and, gradually losing their phagocytic power, appear to develop into fibroblastic cells. In the chronic stage the infected joints show ulceration of the cartilage and synovial membrane, with hyperplasia of the bone marrow and fibrous thickening of the capsule.

The most interesting changes in the heart are the myocardial lesions, which may be briefly described as small foci of necrosis infiltrated and surrounded in most cases by lymphoid cells and macrophages. The location of these lesions in the heart muscle proper, the

arrangement of the cells, and the absence of giant cells distinguish them sharply from Aschoff bodies. In a small percentage of cases bacterial endocarditis or pericarditis occurs.

The lesions produced in other organs are not especially characteristic. Focal necroses are frequently found in the liver, and the kidneys often show foci of round cell infiltration. In the more chronic cases there may be some production of fibrous tissue.

The large endothelioid cells in the heart muscle appear to develop from the vascular endothelium. In this respect my observations agree with those of Evans. In many sections the endothelial sprouts can be seen already developing phagocytic power, as shown by the presence in them of the trypan blue granules.

In the joint exudates the origin of the macrophages is not so clear. One thing is obvious in all the organs; namely, that the injection of trypan blue stimulates an increased production of macrophages.

In the three control rabbits that received no streptococci but were injected with trypan blue alone, the increased number of macrophages was noticeable in all the organs, but strikingly so in the liver and heart muscle.

CONCLUSIONS.

1. Repeated injections into rabbits of non-hemolytic streptococci isolated from human cases of infectious endocarditis or rheumatic fever will produce an acute arthritis in the rabbit similar in most respects to the arthritis of acute rheumatism.

2. Microscopical sections of the joints show a gradual transition from an acute exudative inflammation to advanced organization.

3. Endocarditis and pericarditis occur in a small percentage of cases, and focal lesions in the myocardium consisting of necrosis and the infiltration of cells are frequent. These focal lesions differ considerably from Aschoff's submiliary nodules.

4. Lesions in the kidneys and liver occur but are not characteristic.

5. By means of the vital stain it has been shown that the large endothelioid cells which play a prominent part in the joint and myocardial lesions belong to the group of so called macrophages or wandering cells and probably develop from the vascular endothelium.

BIBLIOGRAPHY.

1. Aschoff, L., *Verhandl. deutsch. path. Ges.*, 1904, viii, 46.
2. Geipel, P., *Deutsch. Arch. klin. Med.*, 1905–06, lxxxv, 75.
3. Fraenkel, E., *Beitr. path. Anat. u. allg. Path.*, 1912, lii, 597.
4. Coombs, C., *Brit. Med. J.*, 1907, ii, 1513; *Quart. J. Med.*, 1908–09, ii, 26.
5. Bracht, E., and Wächter, *Deutsch. Arch. klin. Med.*, 1909, xcvi, 493.
6. Takayasa, R., *Deutsch. Arch. klin. Med.*, 1908–09, xcv, 270.
7. Thalhimer, W., and Rothschild, M. A., *J. Exp. Med.*, 1914, xix, 417, 429, 444.
8. Jackson, L., *J. Infect. Dis.*, 1912, xi, 243; 1913, xii, 364.
9. de Vecchi, B., *Arch. méd. exp. et anat. path.*, 1912, xxiv, 352.
10. Longcope, W. T., *J. Exp. Med.*, 1915, xxii, 793.
11. Coombs, C., Miller, R., and Kettle, E. H., *Lancet*, 1912, ii, 1209.
12. Schloss, O. M., and Foster, N. B., *J. Med. Research*, 1913, xxix, 9.
13. Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.
14. Goldmann, E., *Beitr. klin. Chir.*, 1909, lxiv, Suppl. 192; *Proc. Roy. Soc., Series B*, 1912, lxxxv, 146.
15. Evans, H. M., *Am. J. Physiol.*, 1915, xxxviii, 243.
16. Poynton, F. J., and Paine, A., *Lancet*, 1900, ii, 861, 932.
17. Cole, R. I., *J. Infect. Dis.*, 1904, i, 714.
18. Faber, H. K., *J. Exp. Med.*, 1915, xxii, 615.
19. Christian, H. A., Smith, R. M., and Walker, I. C., *Arch. Int. Med.*, 1911, viii, 468.
20. Ophüls, W., *Proc. Soc. Exp. Biol. and Med.*, 1910–11, viii, 75.
21. Longcope, *J. Exp. Med.*, 1913, xviii, 678.
22. Baehr, G., *J. Exp. Med.*, 1912, xv, 330.
23. Löhlein, M., Über die entzündlichen Verändernugen der Glomeruli der menschlichen Nieren und ihre Bedeutung für die Nephritis, Leipsic, 1907.

EXPLANATION OF PLATES.

Plate 49.

Fig. 1. Rabbit 8. Section through capsule of left shoulder. Subacute arthritis. The membrane, which is considerably thickened, shows internally a layer of necrotic material beneath which the tissue is densely infiltrated with macrophages and leukocytes. Leitz obj. 3, oc. 4.

Fig. 2. Rabbit 5. Focal necrosis in heart muscle. No cellular infiltration has occurred. Leitz obj. 5, oc. 4.

PLATE 50.

FIG. 3. Rabbit 36. Focus in heart muscle, showing necrotic center, surrounded by macrophages and lymphocytes. Vitally stained cells contain blue granules. Counter-stained with aqueous cochineal. Zeiss obj. 5, oc. 4.

PLATE 51.

FIG. 4. Rabbit 12. Focus in heart muscle, showing infiltration of lymphoid cells. Slight necrosis. Leitz obj. 5, oc. 3.

FIG. 5. Rabbit 31. Focus in myocardium showing repair. A few macrophages and lymphoid cells, but connective tissue cells predominate. Leitz obj. 7, oc. 3.

INDEX TO AUTHORS.

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INDEX TO SUBJECTS.

FIG. 1.

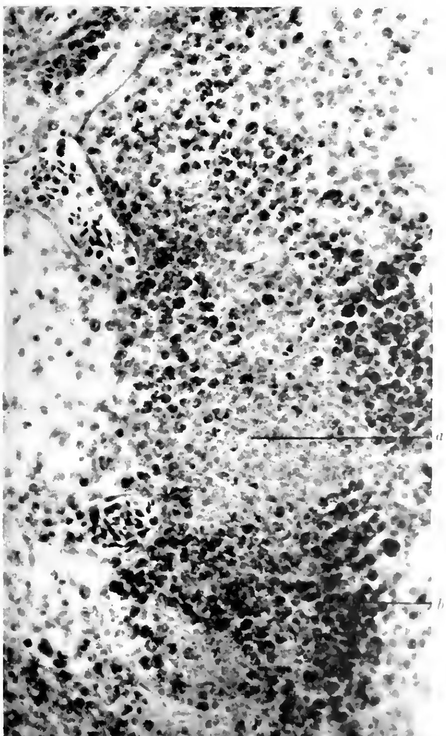


FIG. 2.

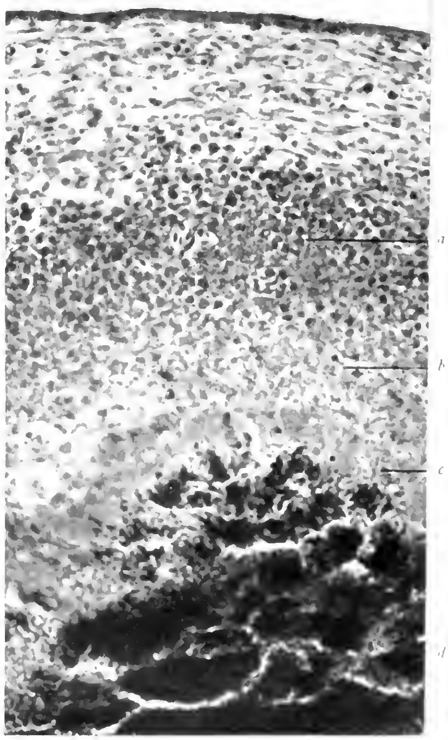


FIG. 3.

Murphy: Adult Chicken Organ Grafts on Chick Embryo.

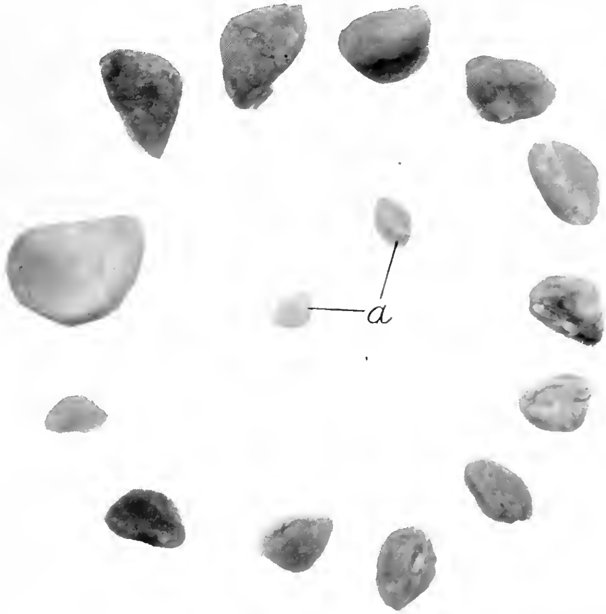


FIG. 4.

FIG. 5.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

Fig. 6.

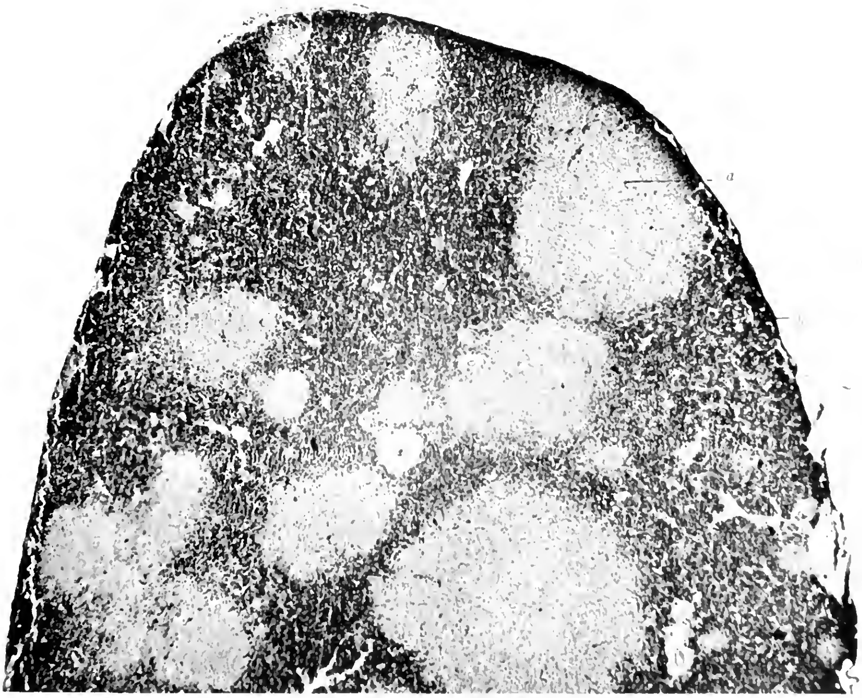


Fig. 7.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo)

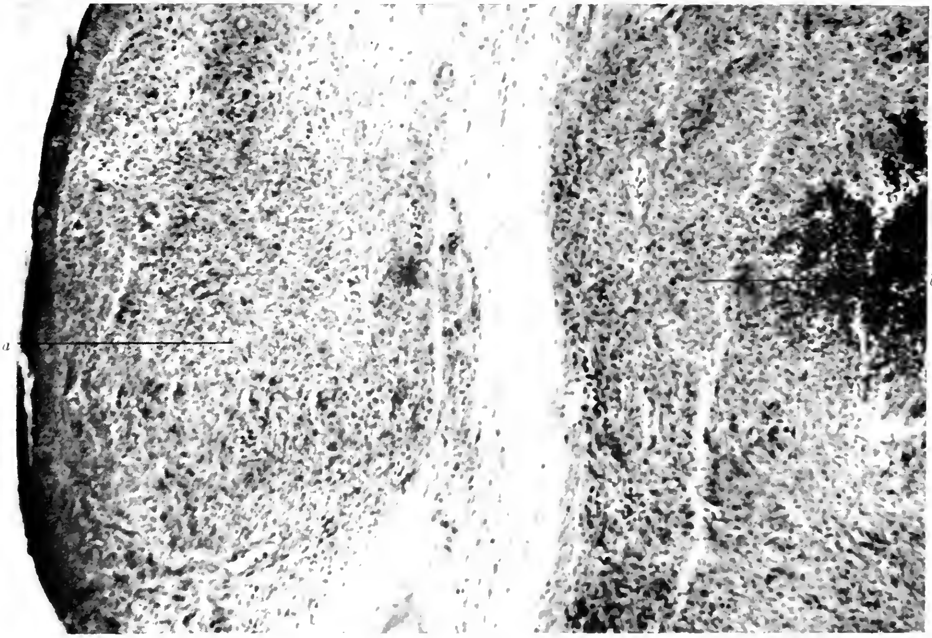


Fig. 8.

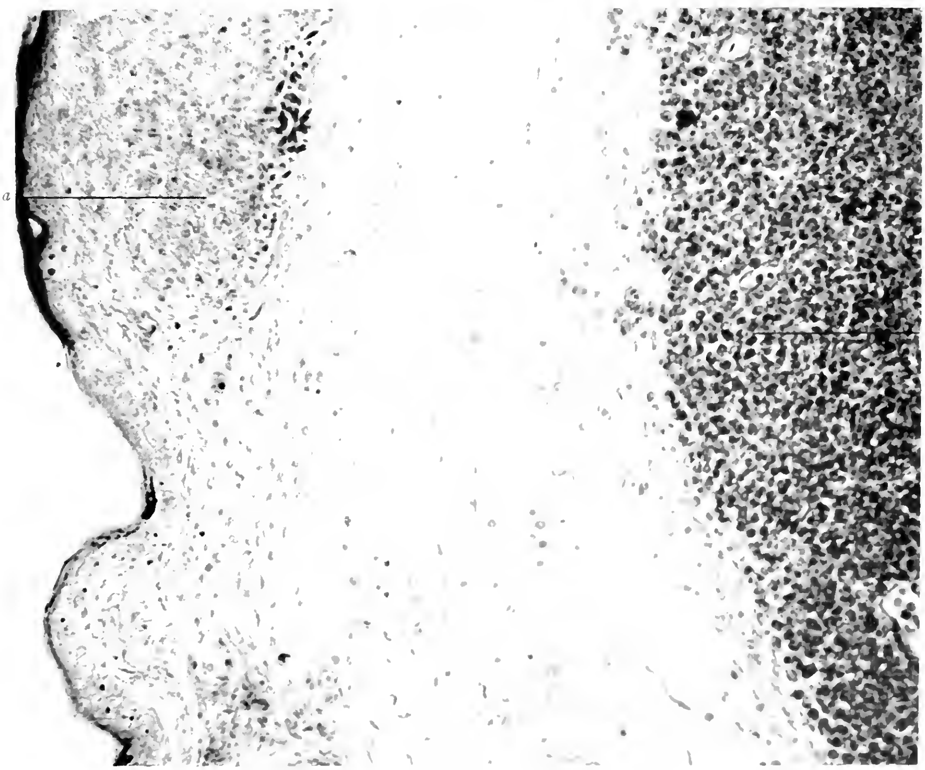


Fig. 9.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo

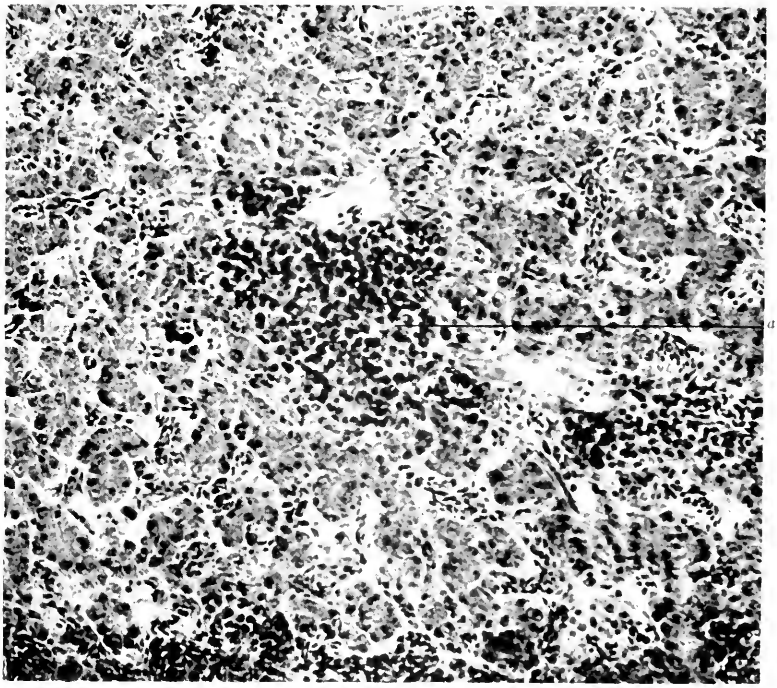


FIG. 10.

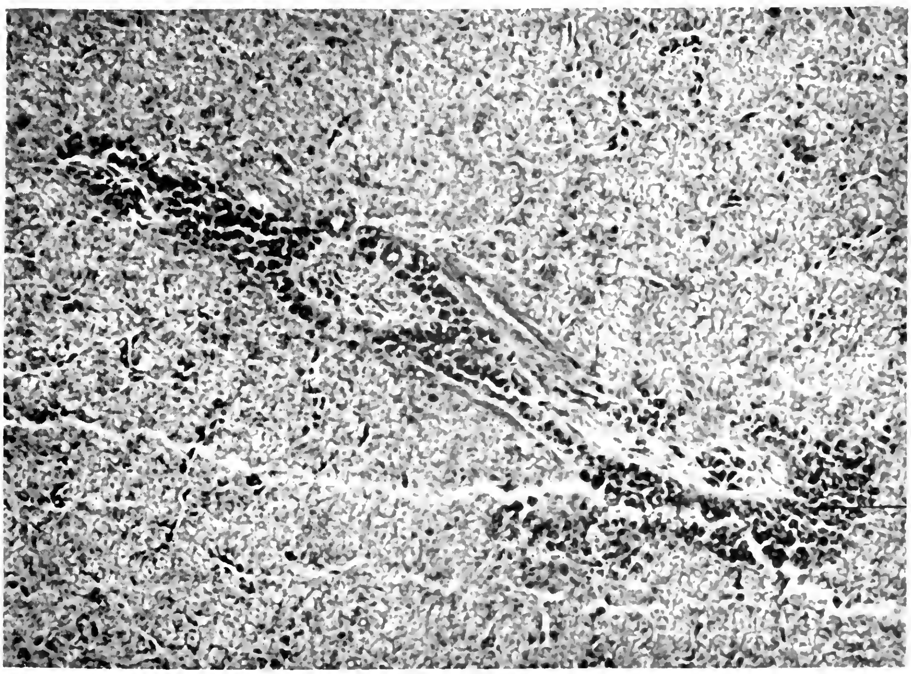
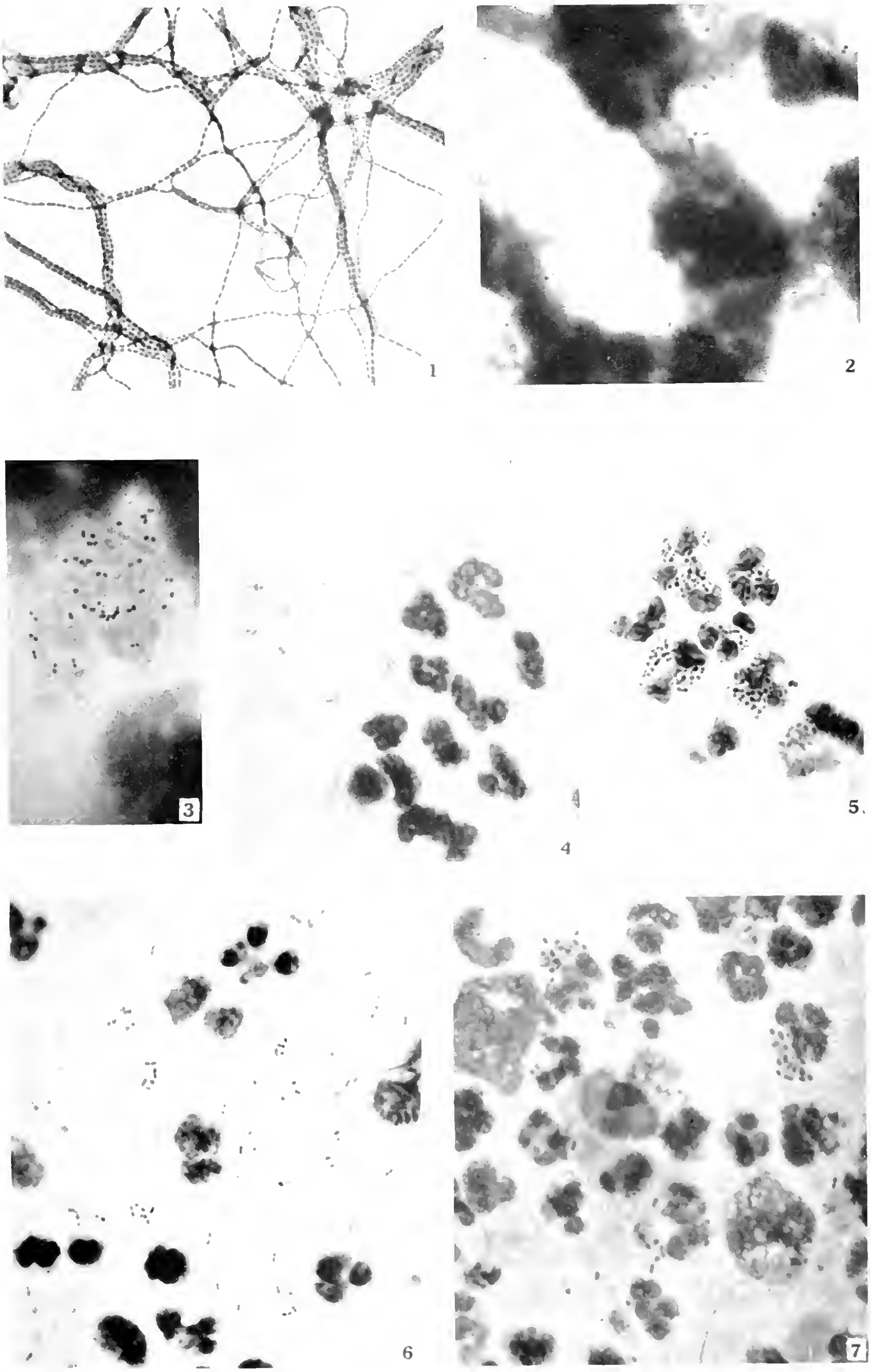
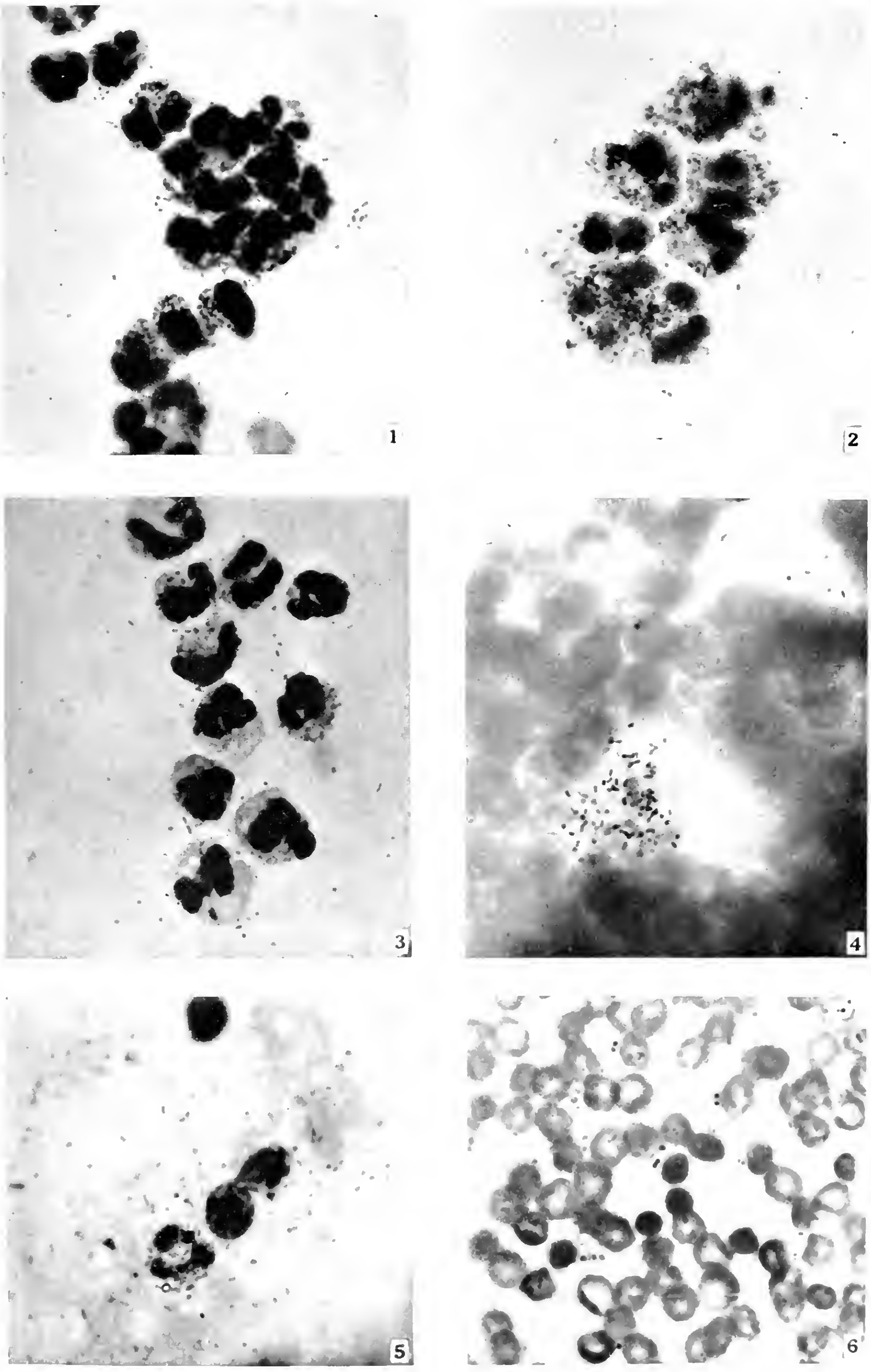


FIG. 11.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)



(Bull: Immunity in Pneumococcus Infection.)



Bull: Agglutination of Bacteria *in Vivo*

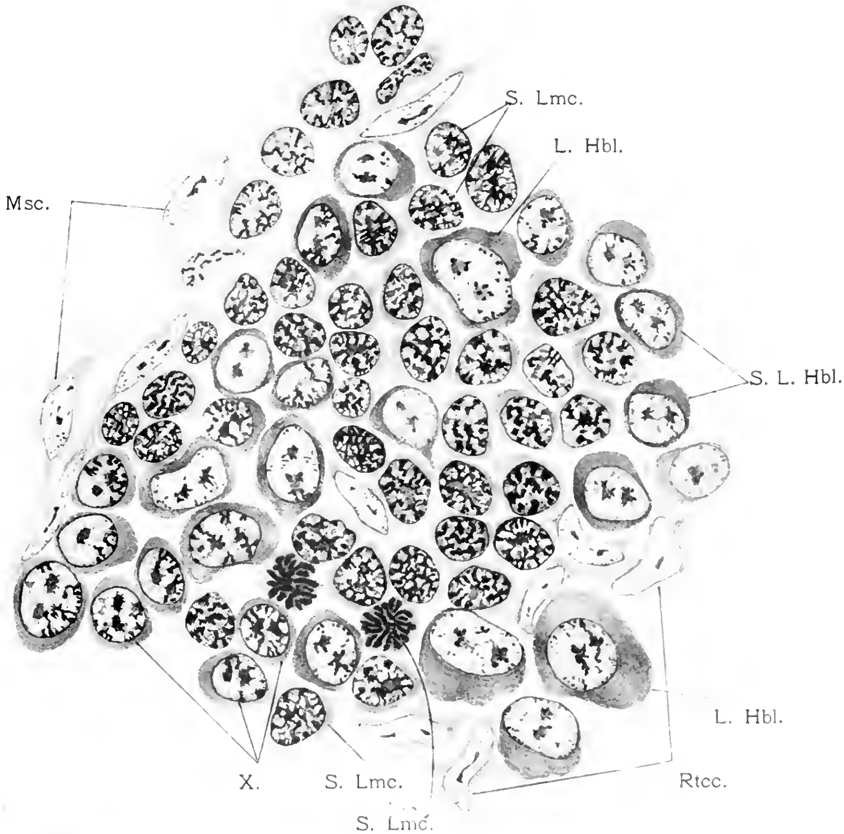
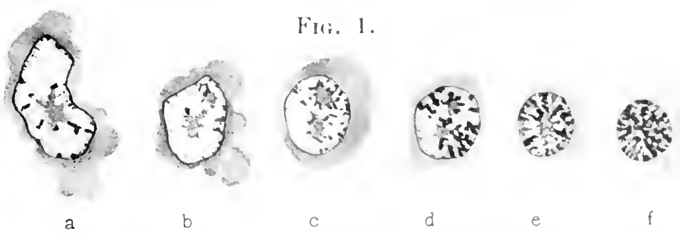


FIG. 1.



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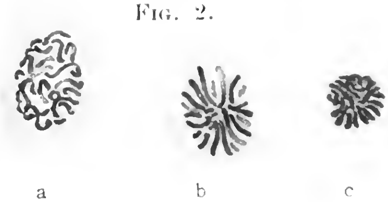
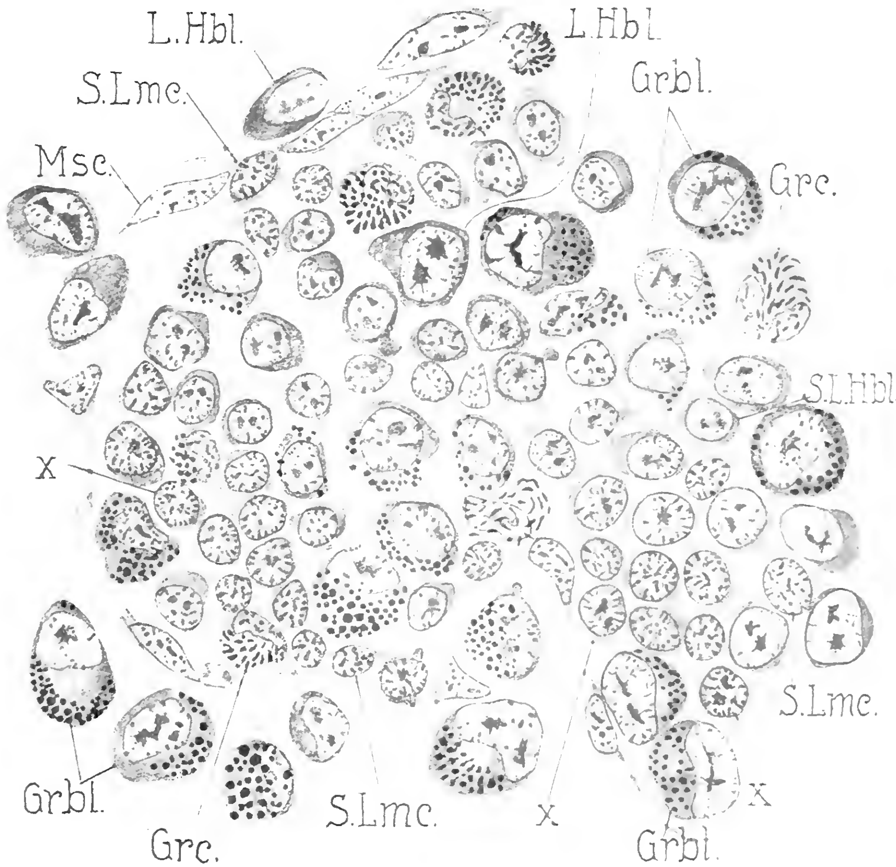


FIG. 3.

Vera Danchakoff fec. (Danchakoff: Differentiation of Cells.)

HELIOTYPE CO., BOSTON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L.Hbl.
Grbl.
S.Lmc.
Msc.
Grc.
S.L.Hbl.
X
S.Lmc.
X
Grbl.
S.Lmc.
X
Grc.
Grbl.

FIG. 4.

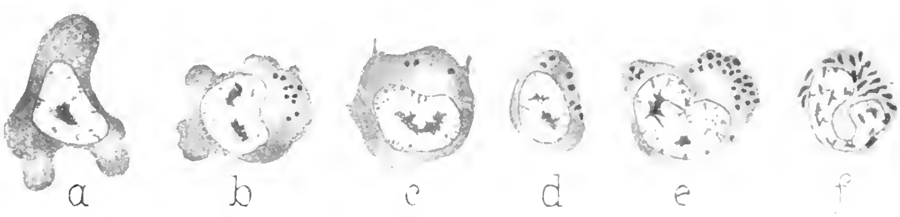

a
b
c
d
e
f

FIG. 5.

FIG. 6.

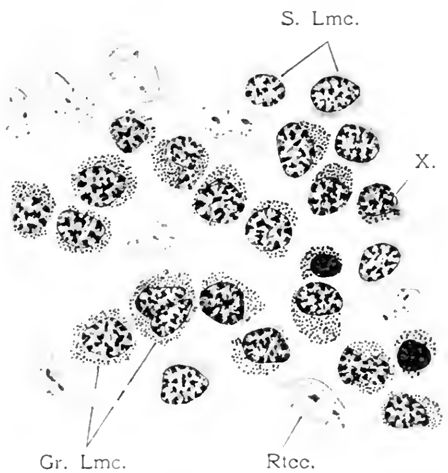


FIG. 7.

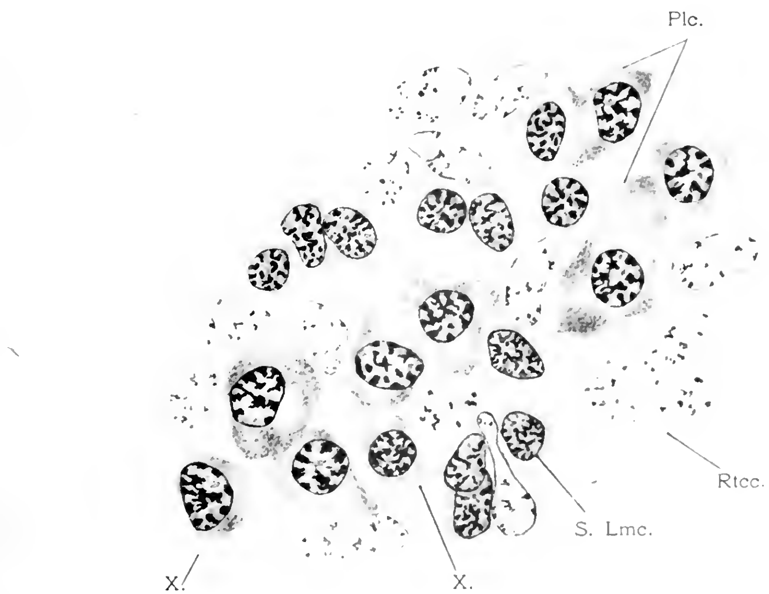


FIG. 8.

Vera Danchakoff fec.

(Danchakoff: Differentiation of Cells.)

HELIOTYPE CO., BOSTON

FIG. 1.

(Koga: Chemotherapy of Tuberculosis.)

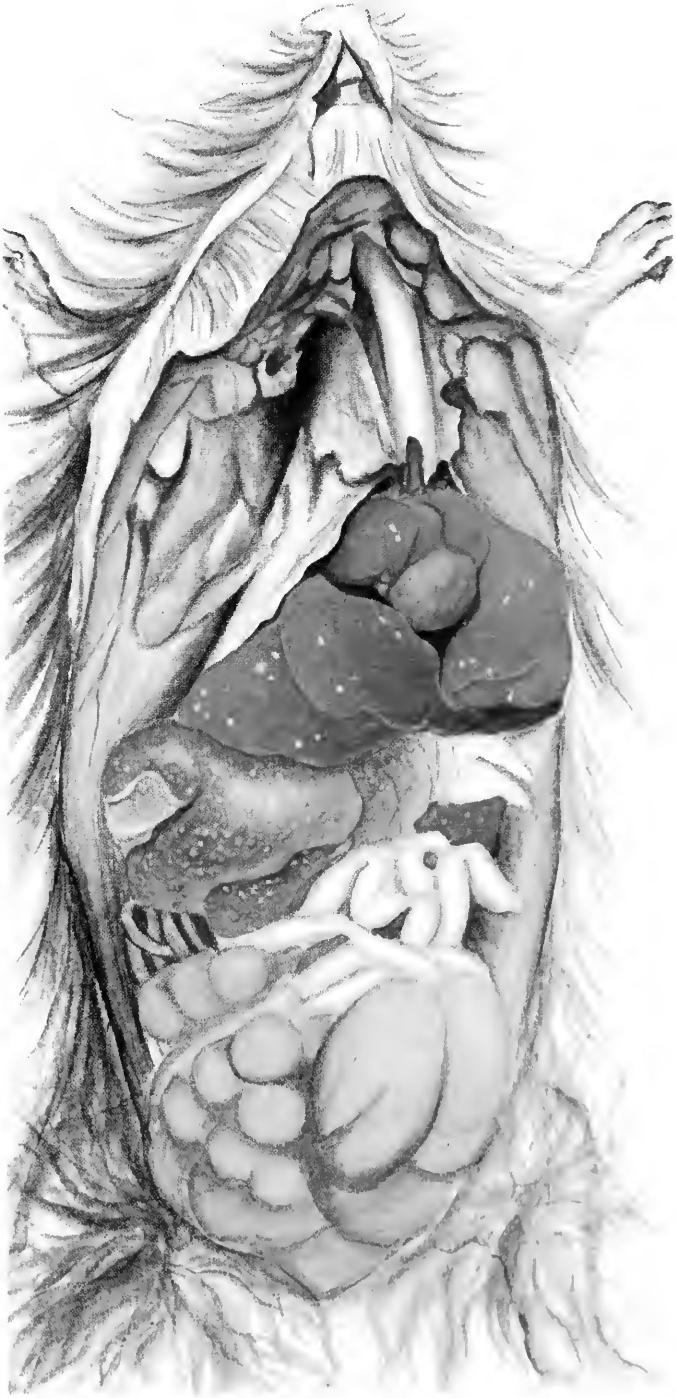


FIG. 2.

(Koga: Chemotherapy of Tuberculosis.)

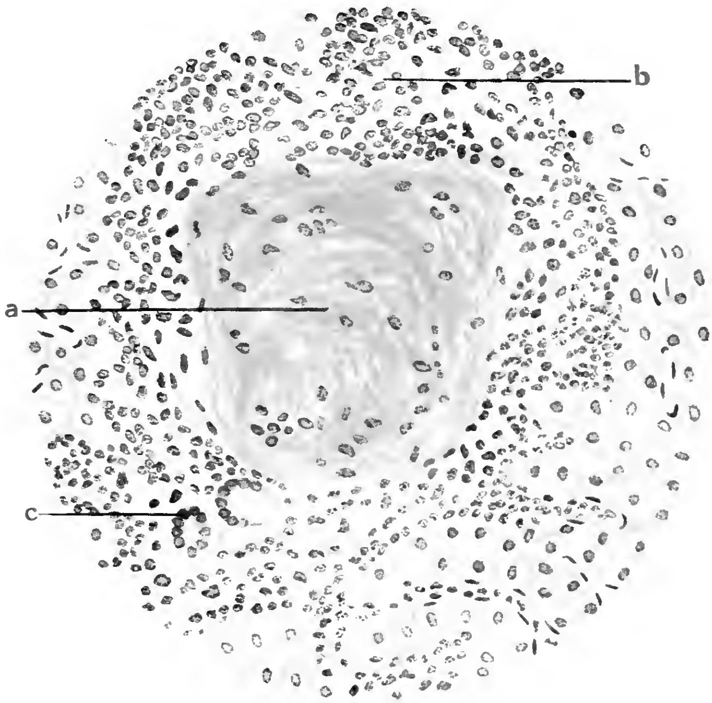


FIG. 3.

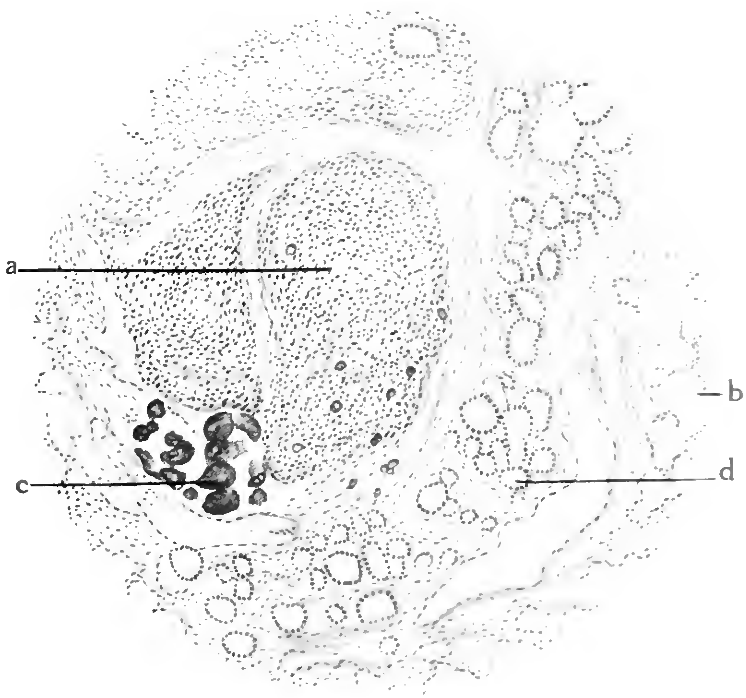


FIG. 4.

(Koga: Chemotherapy of Tuberculosis.)

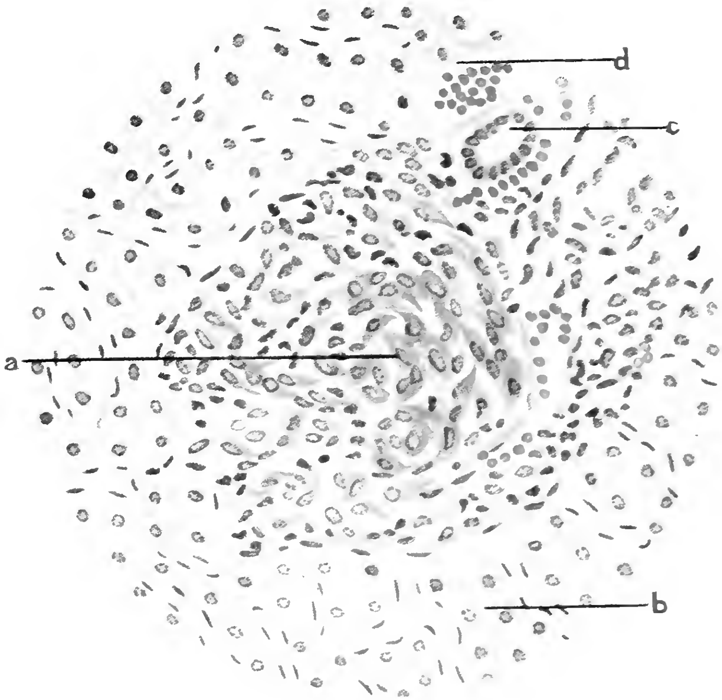


FIG. 5.

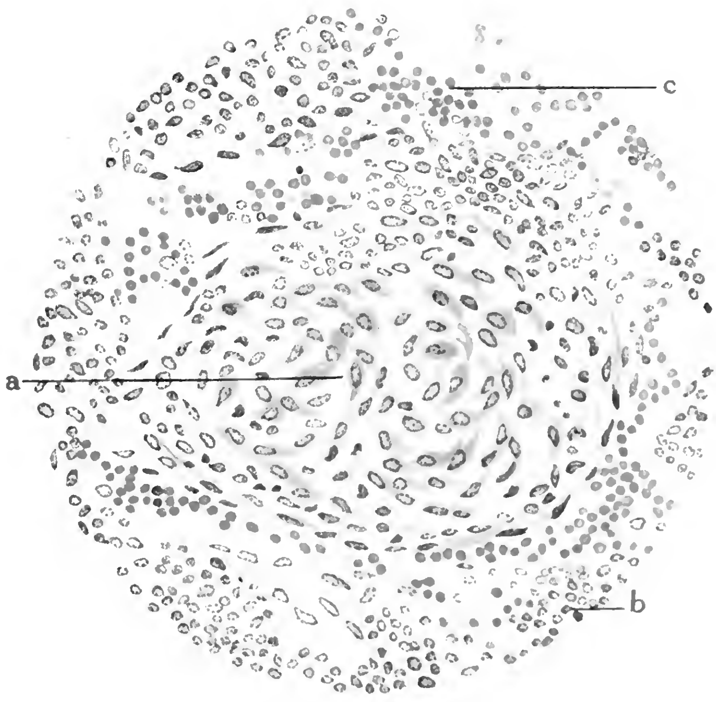


FIG. 6.

(Koga: Chemotherapy of Tuberculosis.)

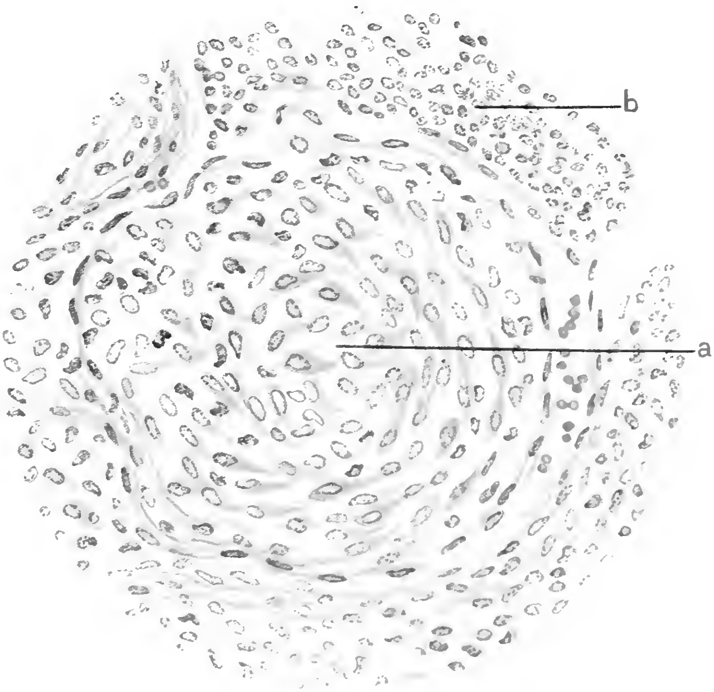
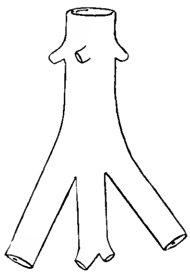


FIG. 7.

(Koga: Chemotherapy of Tuberculosis



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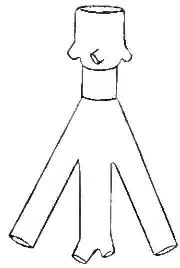

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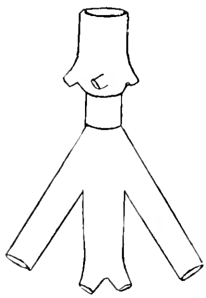

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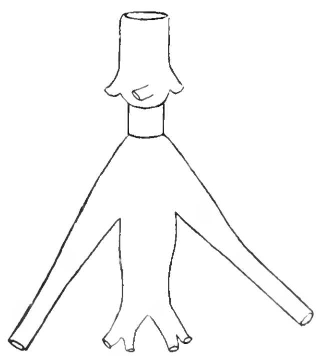
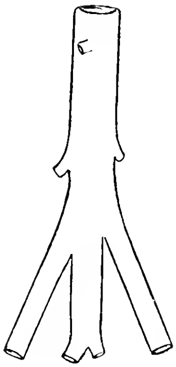


4

(Halsted: Circumscribed Dilation of an Artery.)



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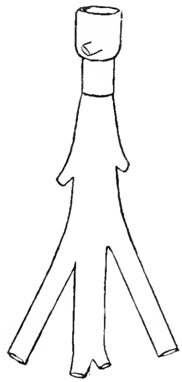

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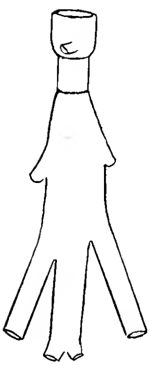

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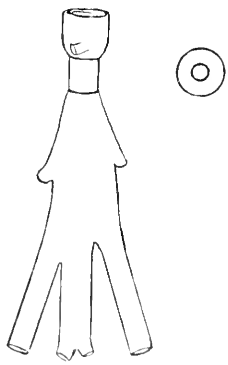

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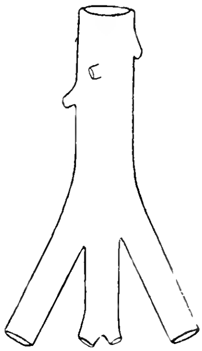
(Halsted: Circumscribed Dilation of an Artery.)



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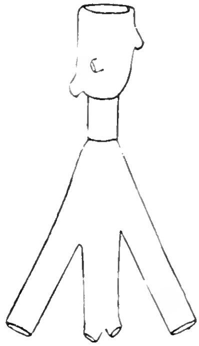

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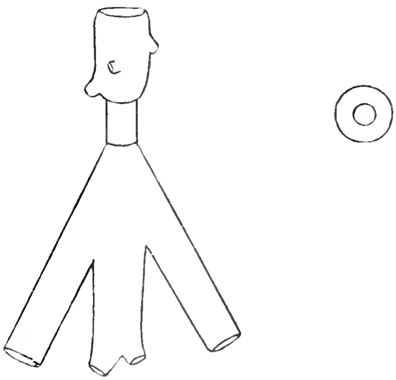

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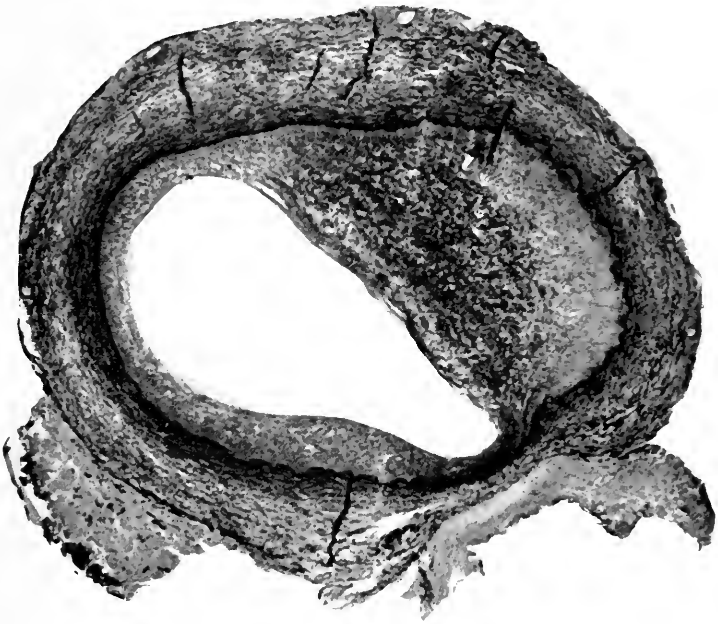
(Halsted: Circumscribed Dilatation of an Artery.)



12

13

(Halsted: Circumscribed Dilation of an Artery.)

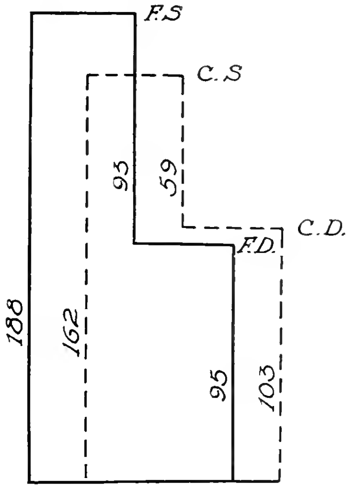


FIG. 1.

F.S. 188. F.D. 95. Pulse pressure 93.
C.S. 162. C.D. 103. Pulse pressure 59.

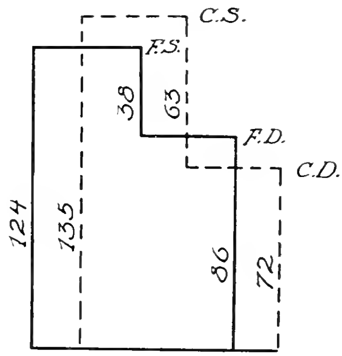


FIG. 2.

F.S. 124. F.D. 86. Pulse pressure 38.
C.S. 135. C.D. 72. Pulse pressure 63.

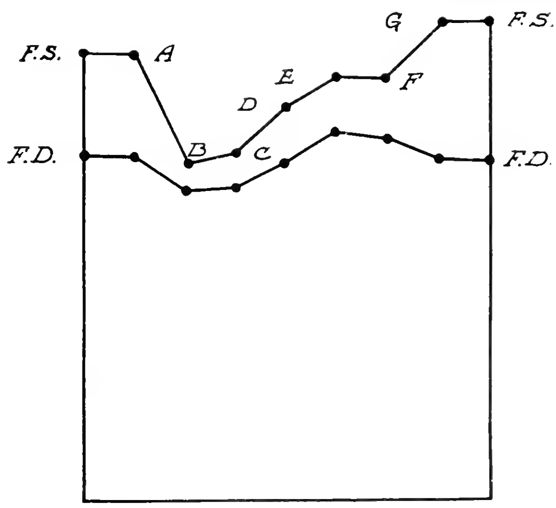


FIG. 3.

A. Without band 175, 135.
B. Immediately after band was applied 131, 121.
C. 5 minutes later 136, 123.
D. 25 minutes later 155, 133.
E. 35 minutes later 167, 144.
F. 35 + minutes later 167, 143.
G. Band removed 189, 138.

FIG. 4.

FIG. 5.

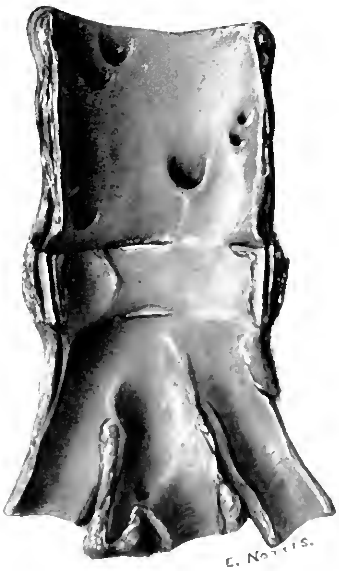


FIG. 6.

(Reid: Partial Occlusion of the Aorta.)

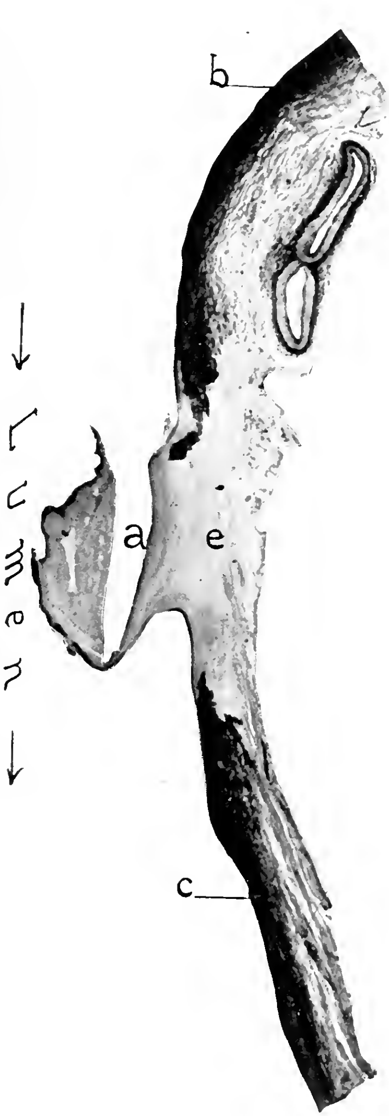


FIG. 7.

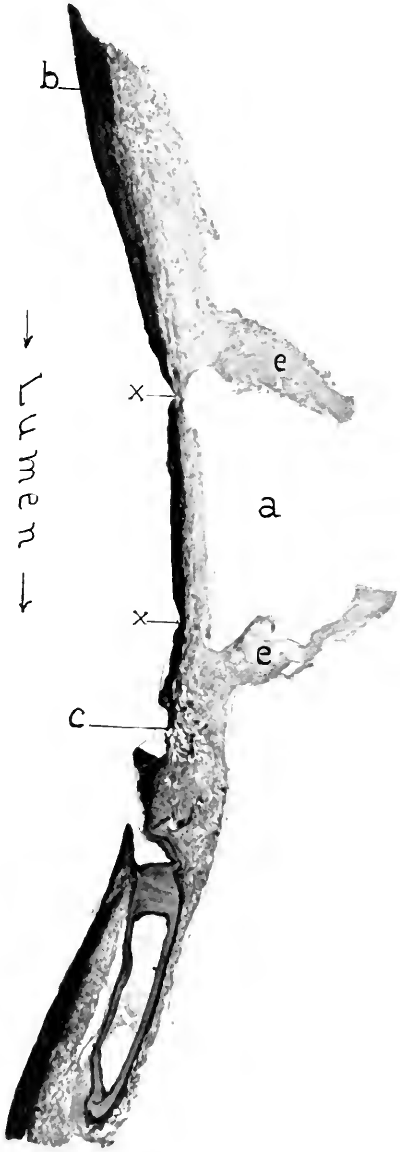


FIG. 8.

(Reid: Partial Occlusion of the Aorta.)

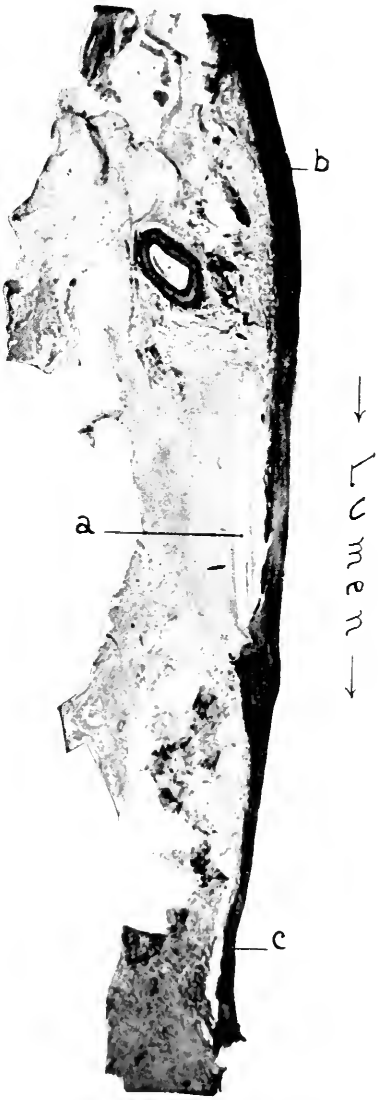


FIG. 9.

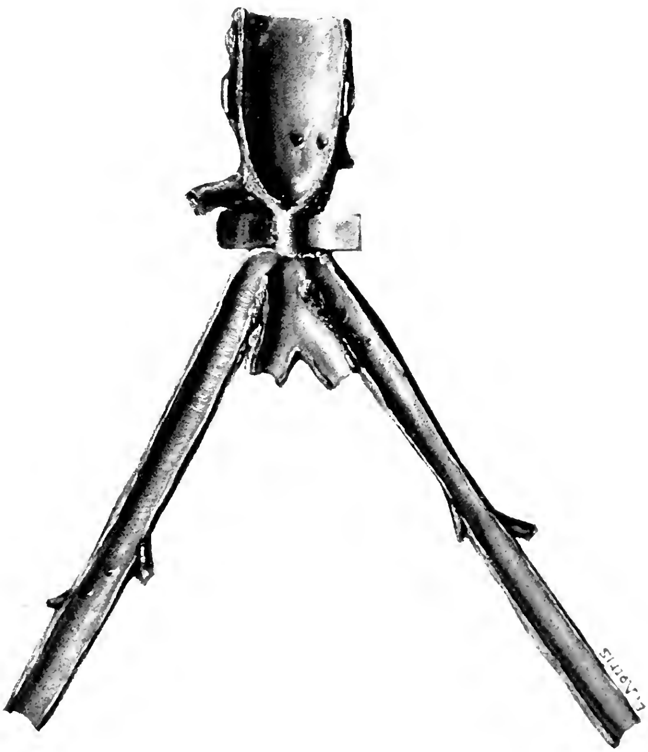


FIG. 10.

(Reid: Partial Occlusion of the Aorta.)

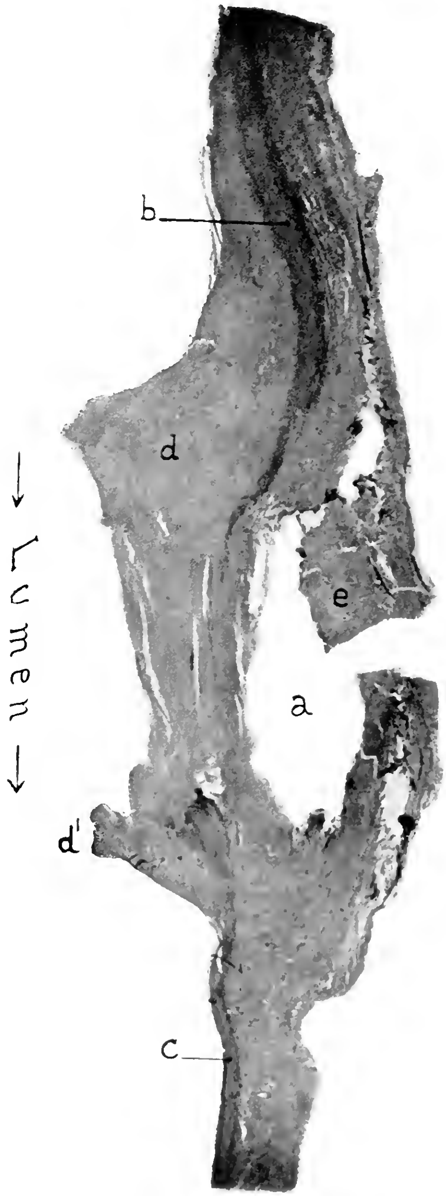


FIG. 11.

(Reid: Partial Occlusion of the Aorta.)

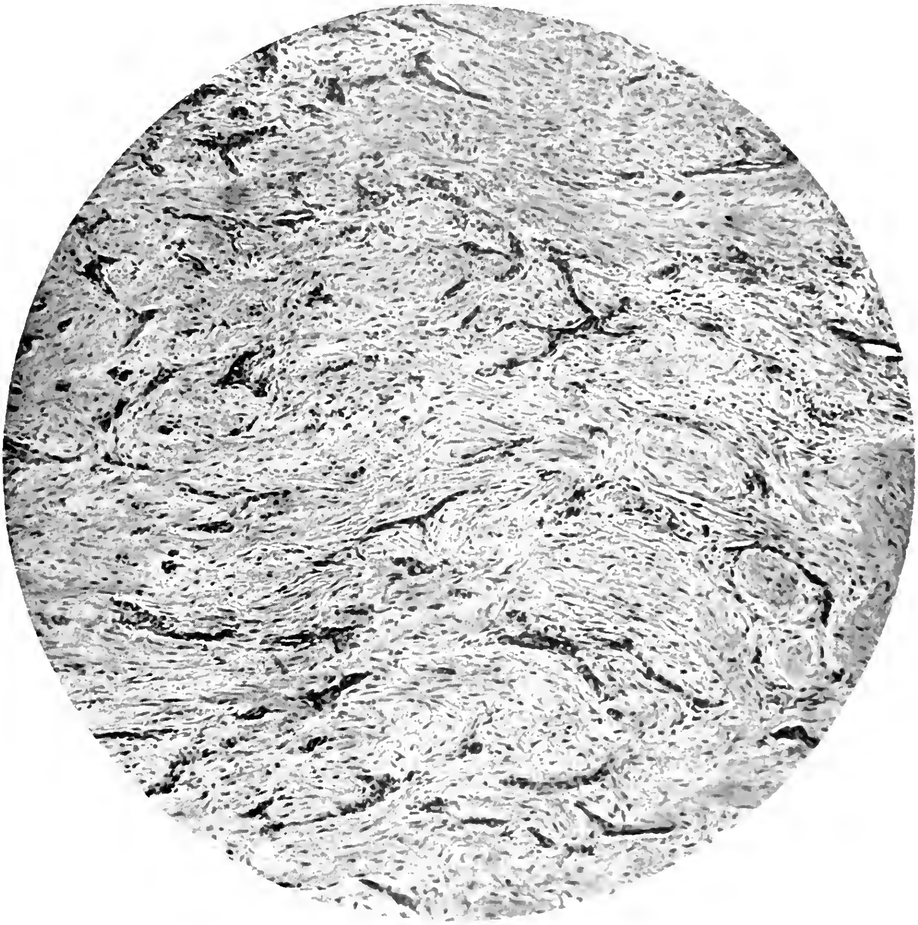
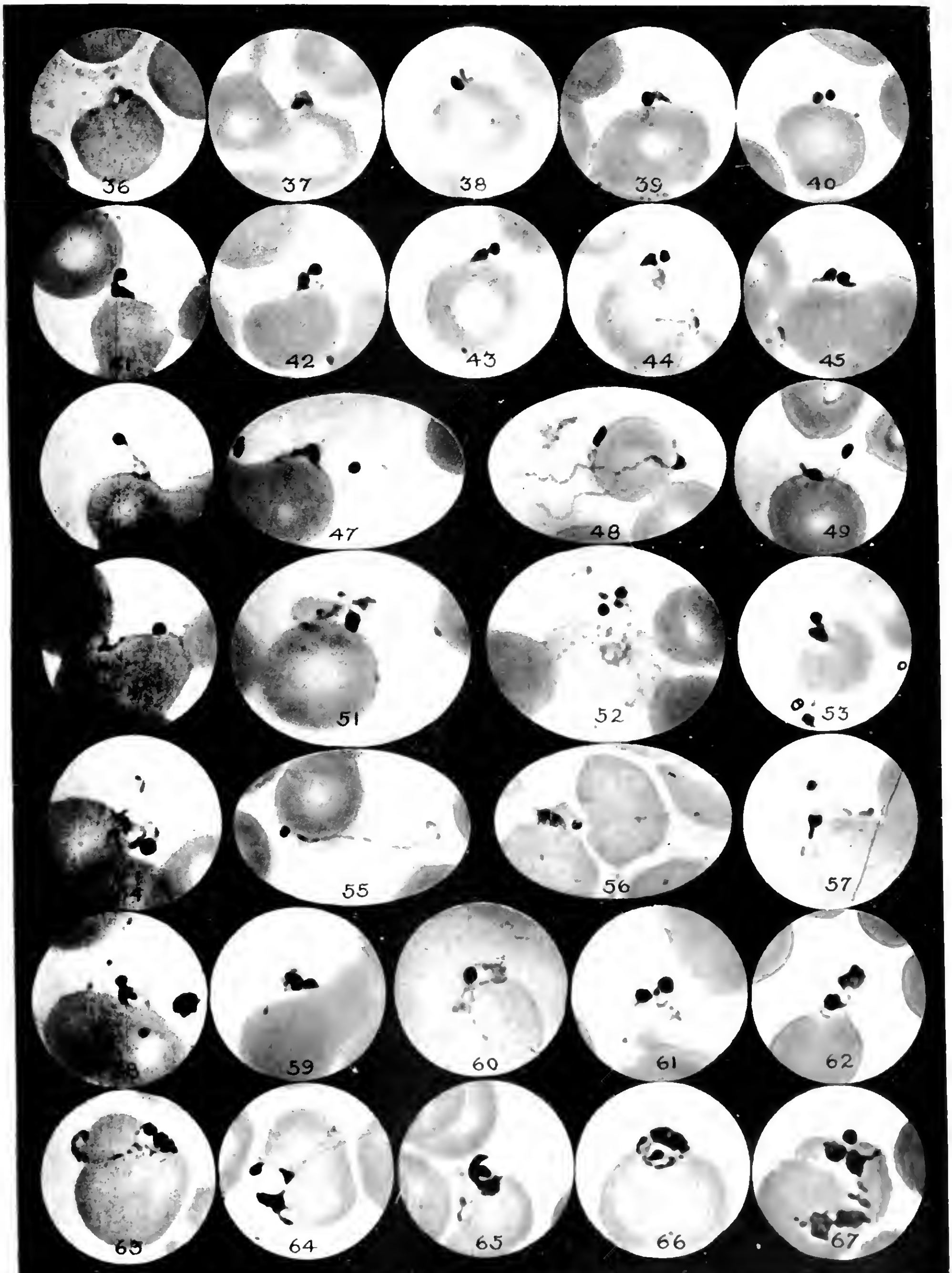


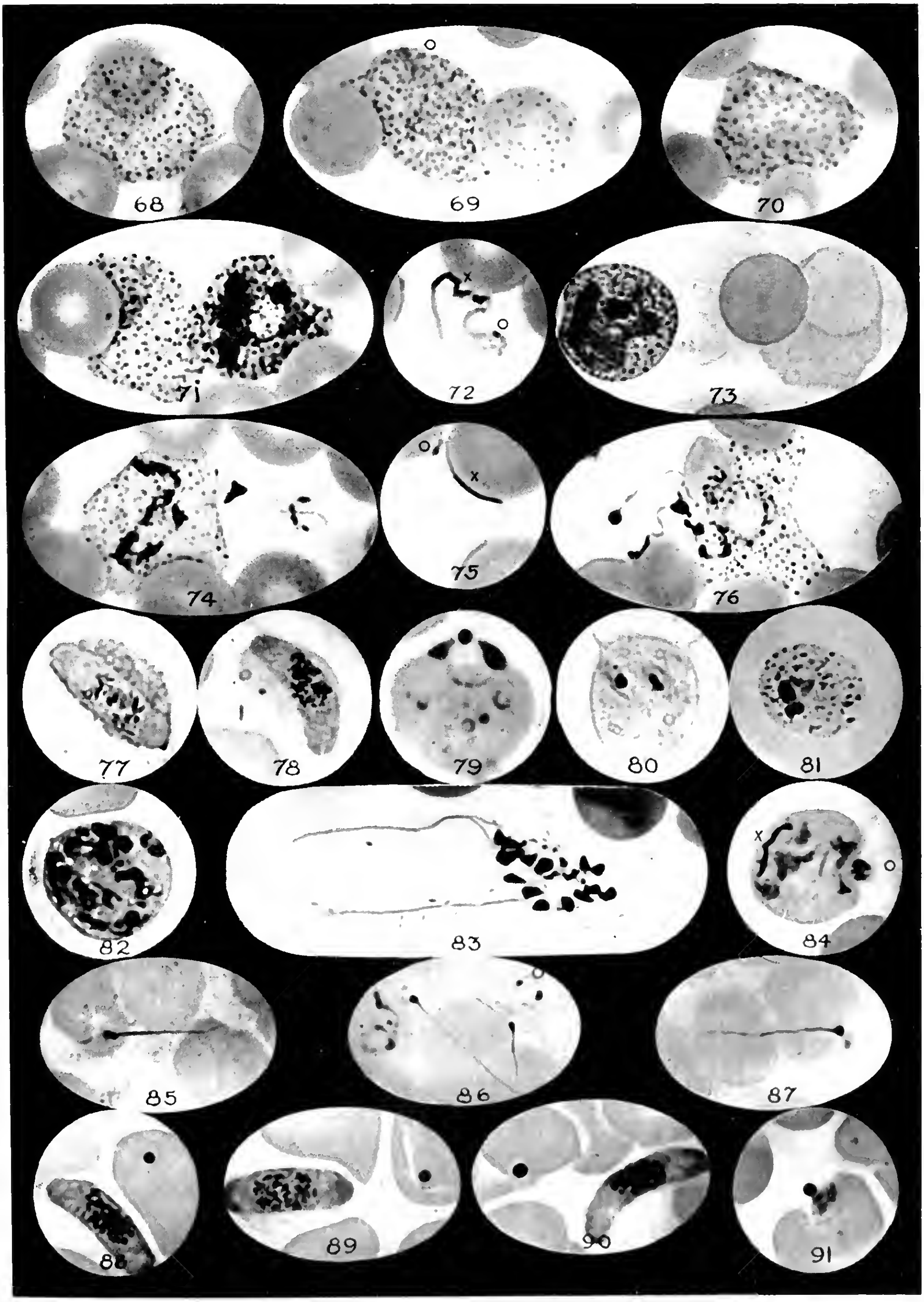
FIG. 12.

(Reid: Partial Occlusion of the Aorta.)

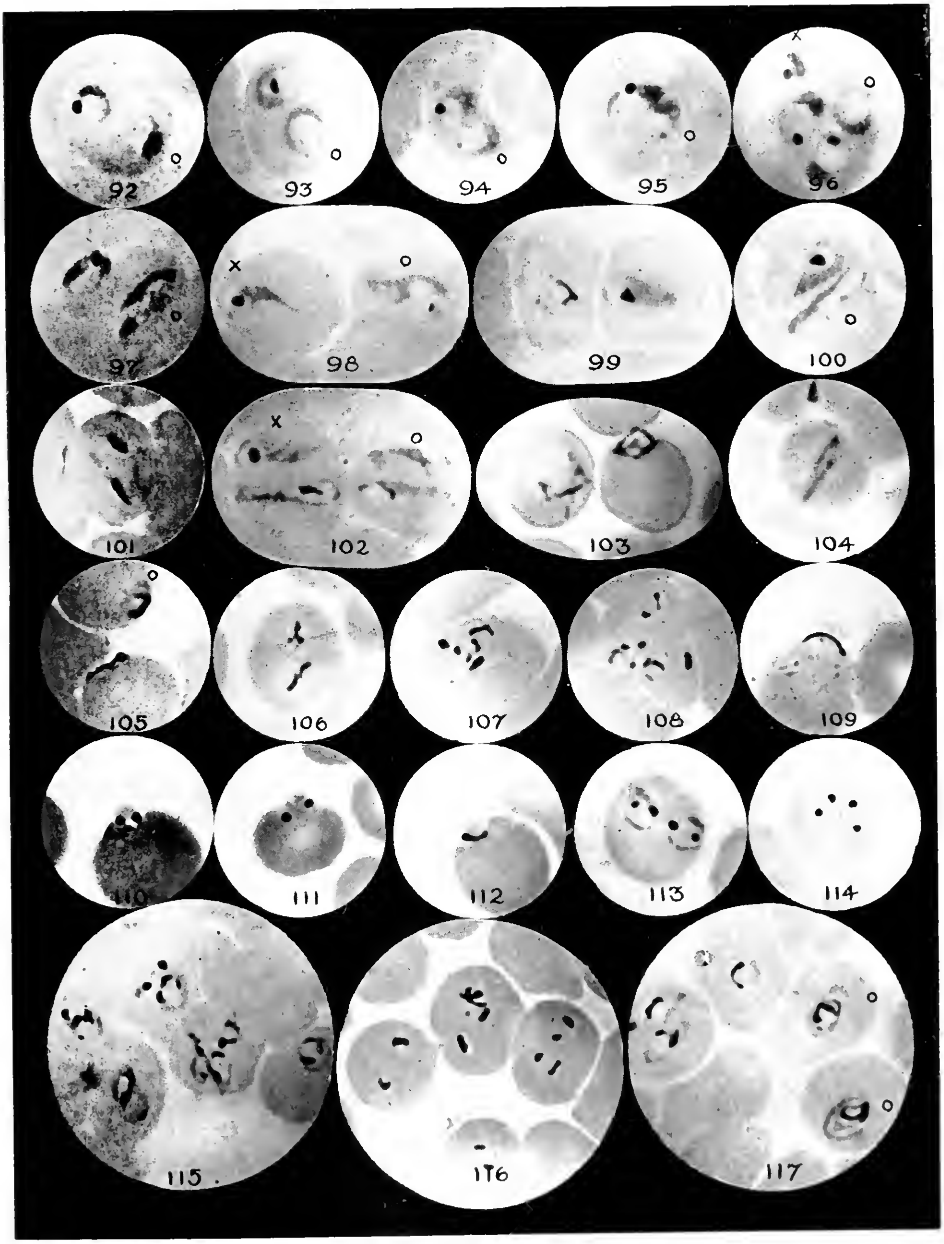
Lawson: Distortion of the Malarial Parasite.



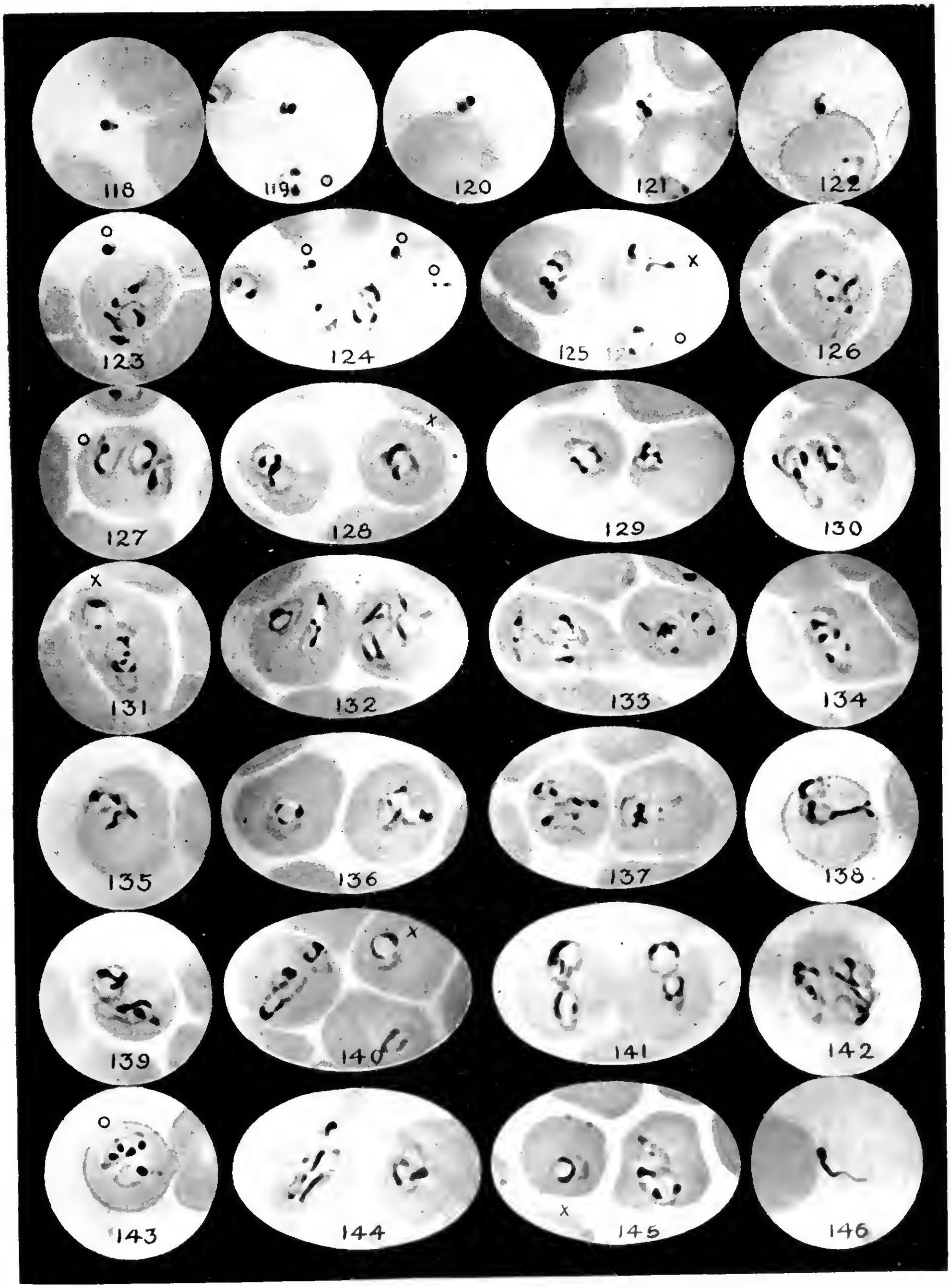
(Lawson: Distortion of the Malarial Parasite.)



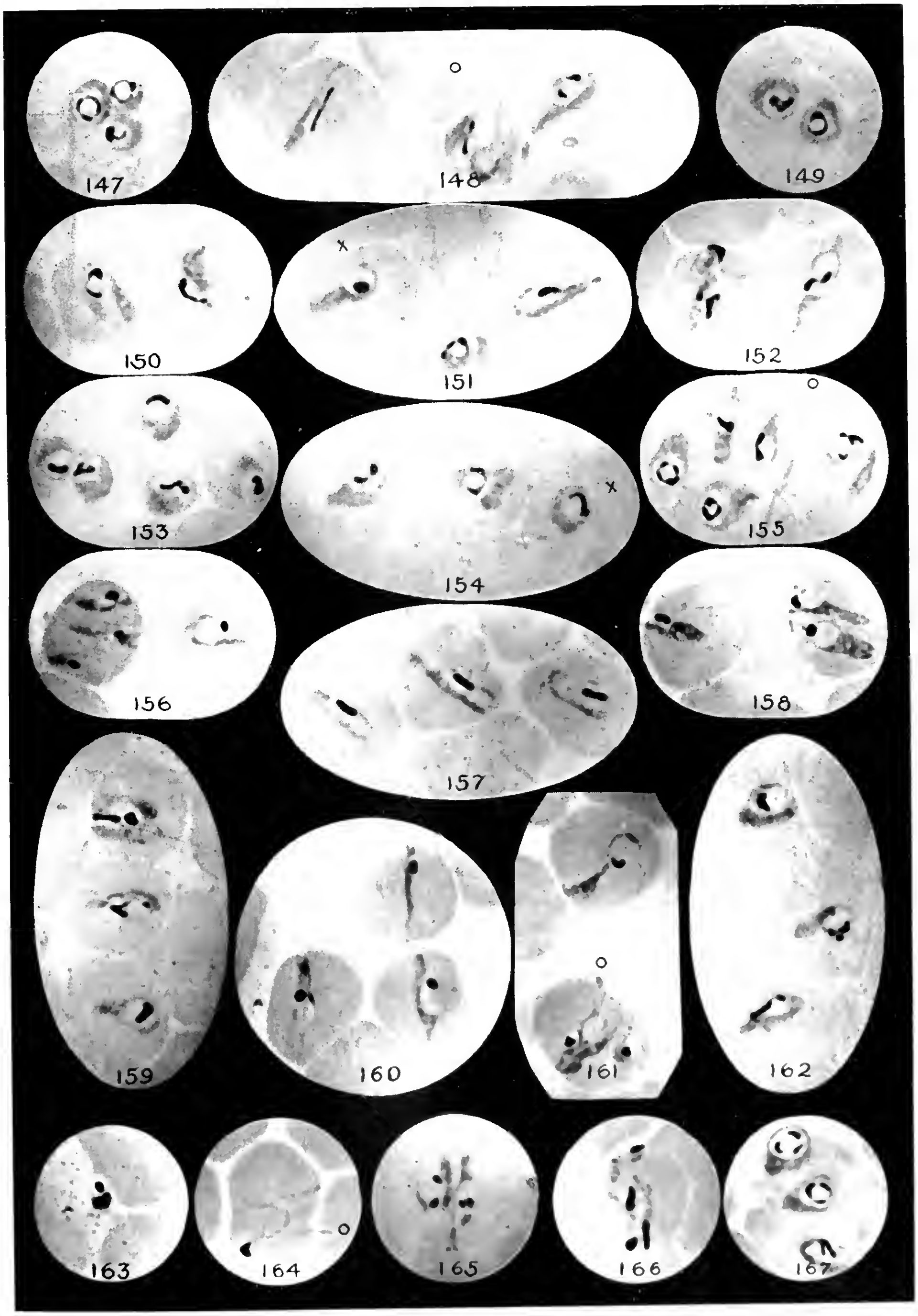
Lawson: Distortion of the Malarial Parasite.



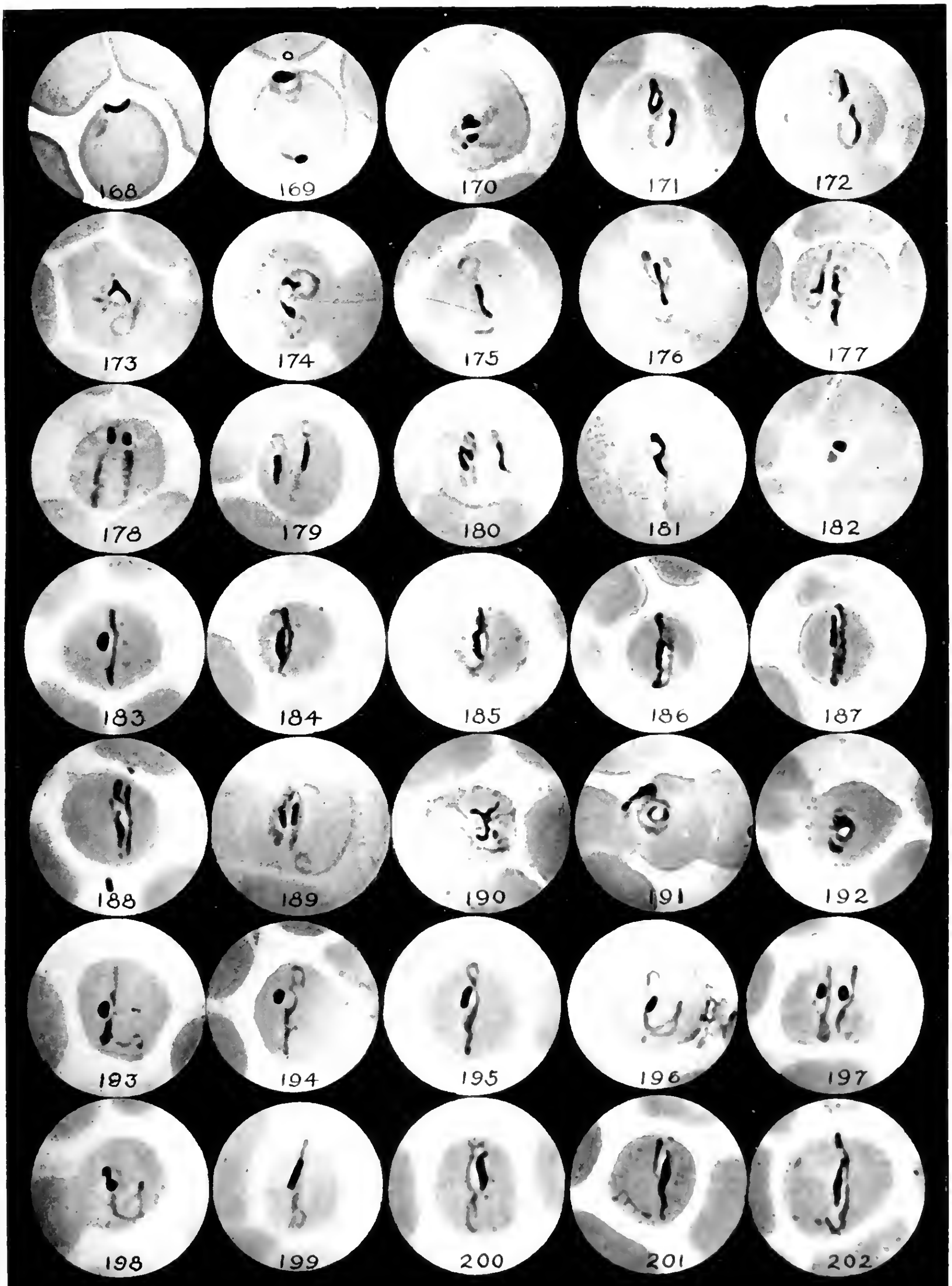
(Lawson: Distortion of the Malarial Parasite.)



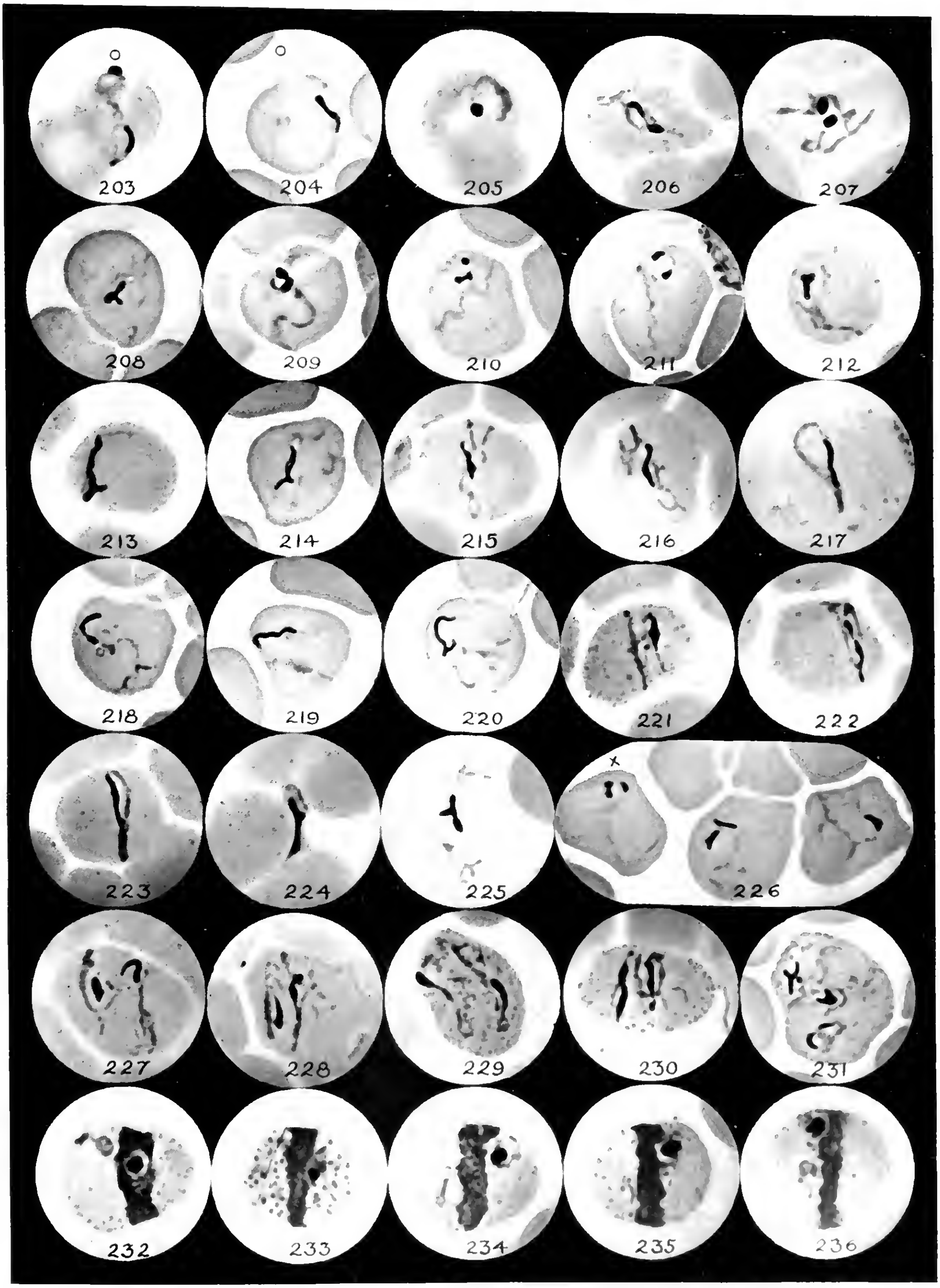
Lawson: Distortion of the Malarial Parasite.



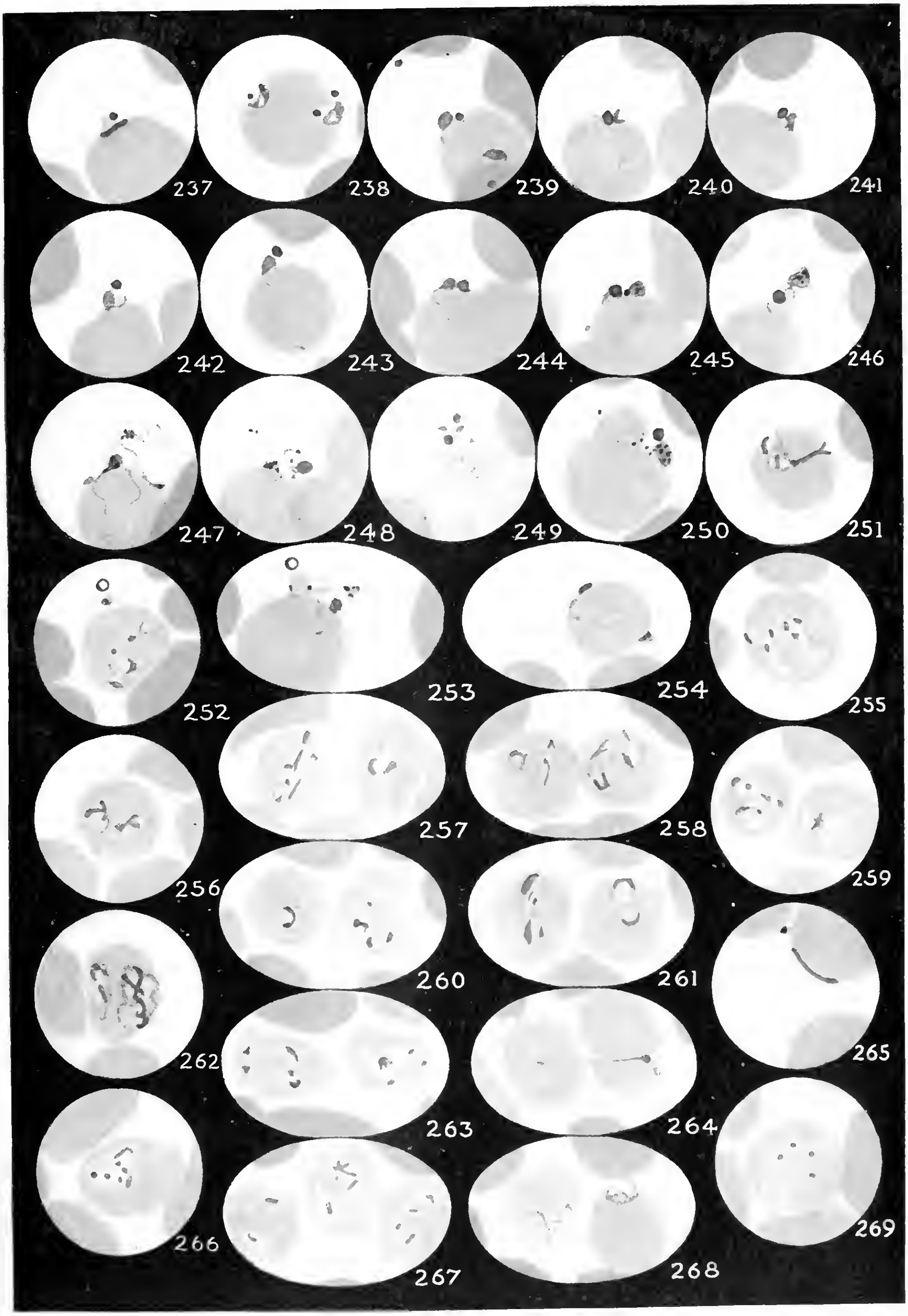
Lawson: Distortion of the Malarial Parasite.



Lawson: Distortion of the Malarial Parasite.



Lawson: Distortion of the Malarial Parasite



(Lawson: Distortion of the Malarial Parasite.)

FIG. 1.

FIG. 2.

Mann: Gastric Ulcers Following Adrenalectomy

FIG. 3.

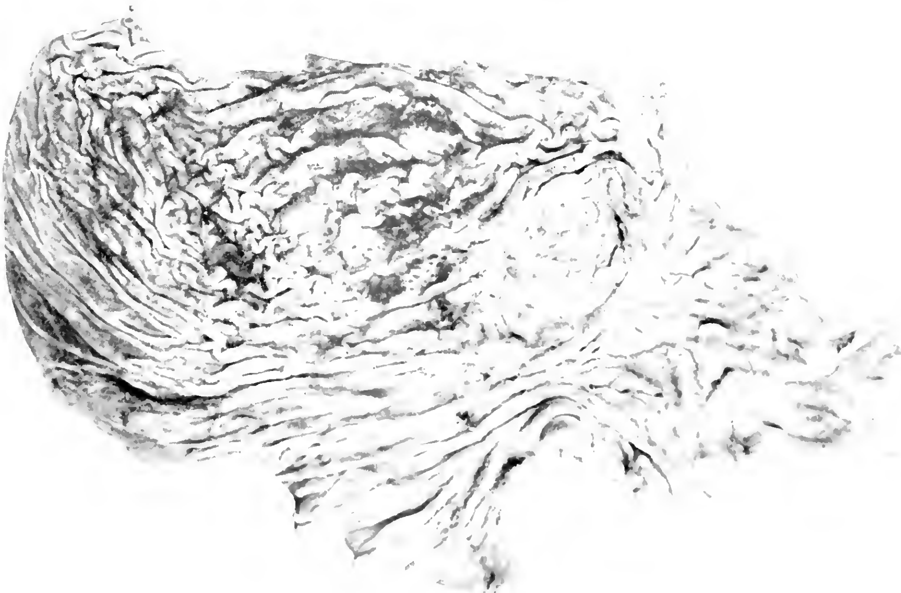


FIG. 4.

(Mann: Gastric Ulcers Following Adrenalectomy.)

Series A.

FIG. 1.

(Graham: [illegible] and the Thyroid Gland.)

Series B.

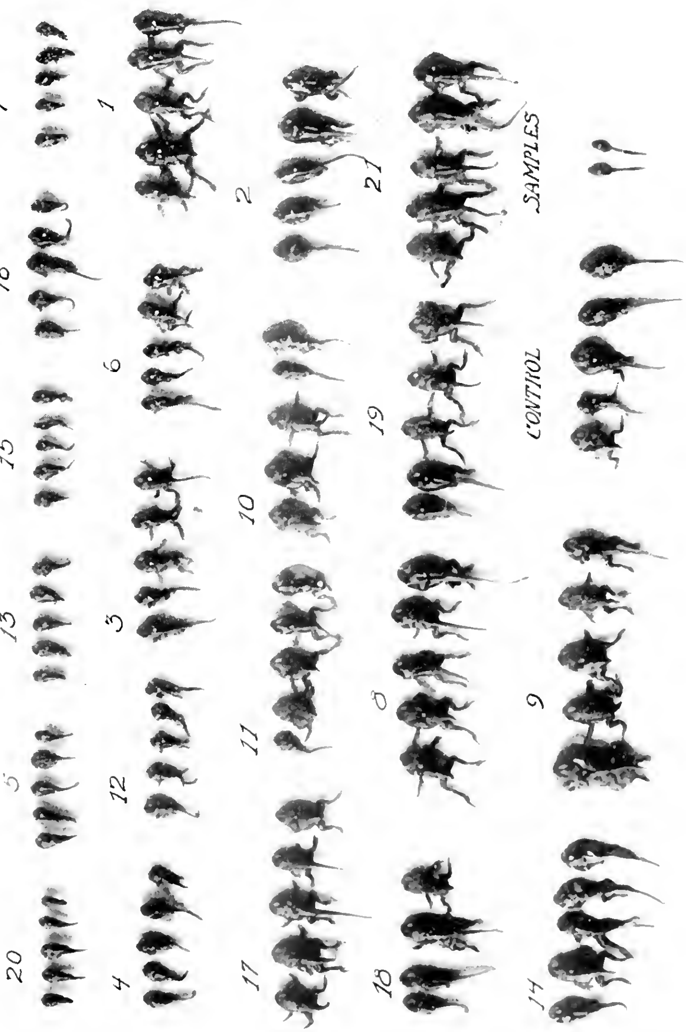


Fig. 2.

Series C.

Samples. Control. 14

May 25. June 10. June 10.

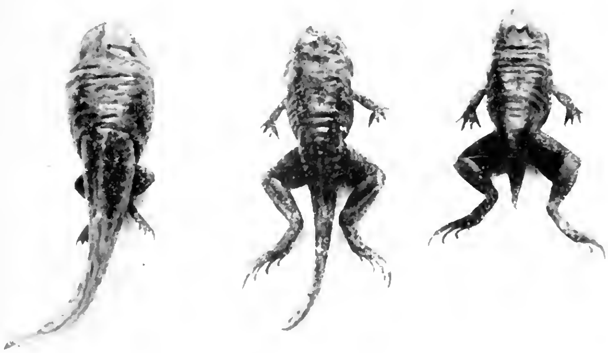
12 15 20

June 10. June 5. June 5. June 4. June 4.

FIG. 3.

Series D.

Sample. Control. 20



Sample. Control. 20

FIG. 4.

(Graham: Adenomata of the Thyroid Gland.)

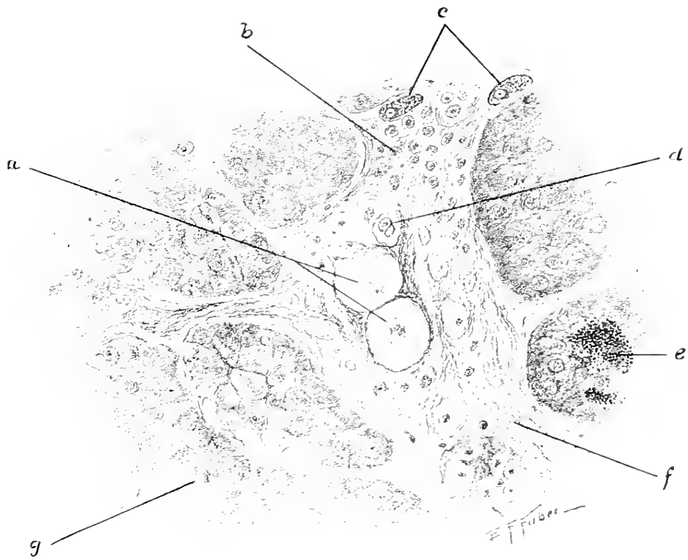


Fig. 1.

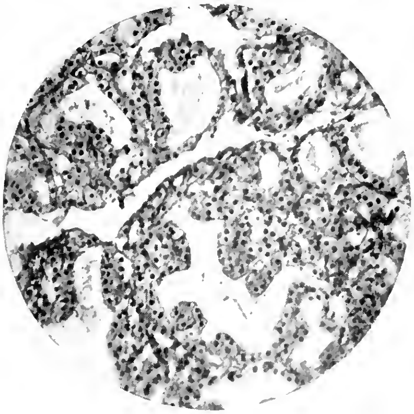


FIG. 1.

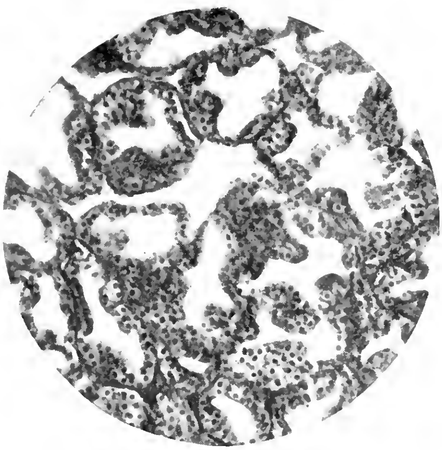


FIG. 2.

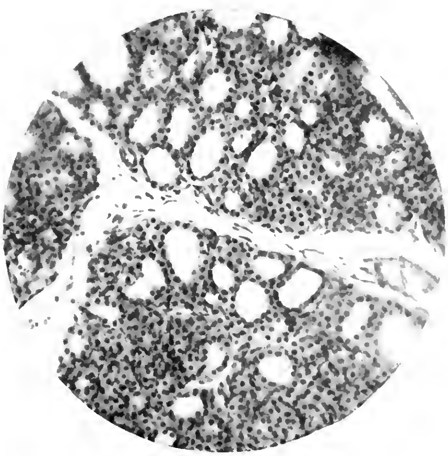


FIG. 3.

FIG. 4.

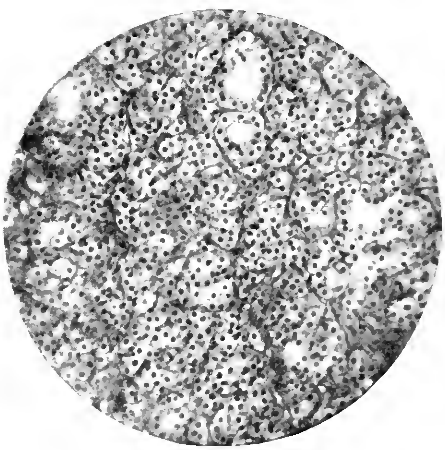


FIG. 5.

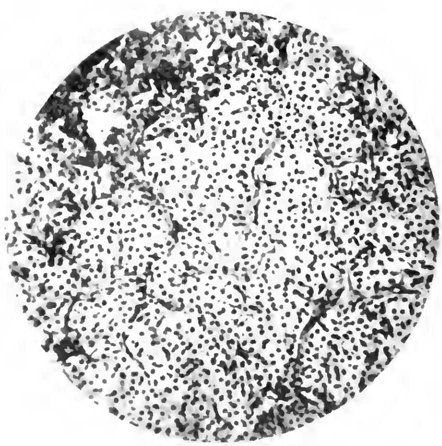


FIG. 6.

(Tanberg: Thyroid and Parathyroid Glands.)

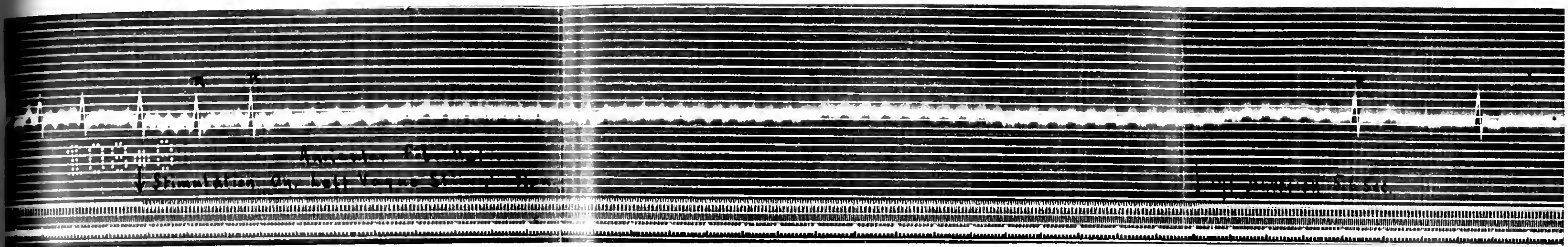


FIG. 1.

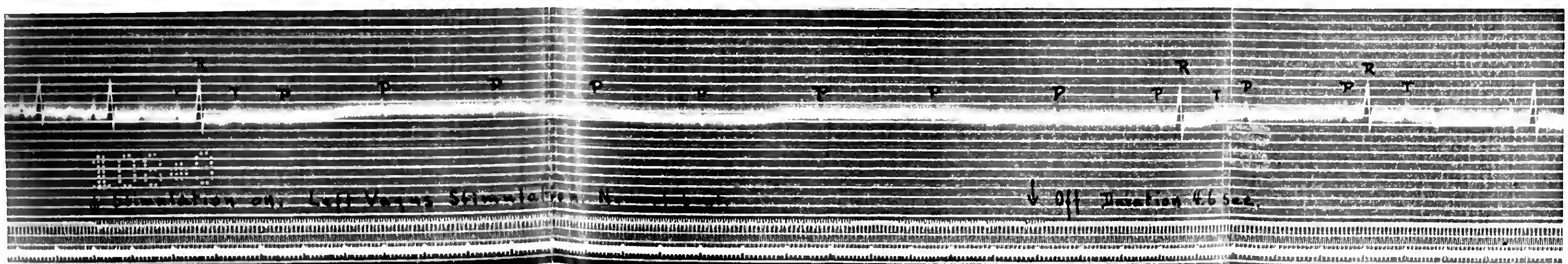


FIG. 2.

(Robinson: Conduction between Auricles and Ventricles.)

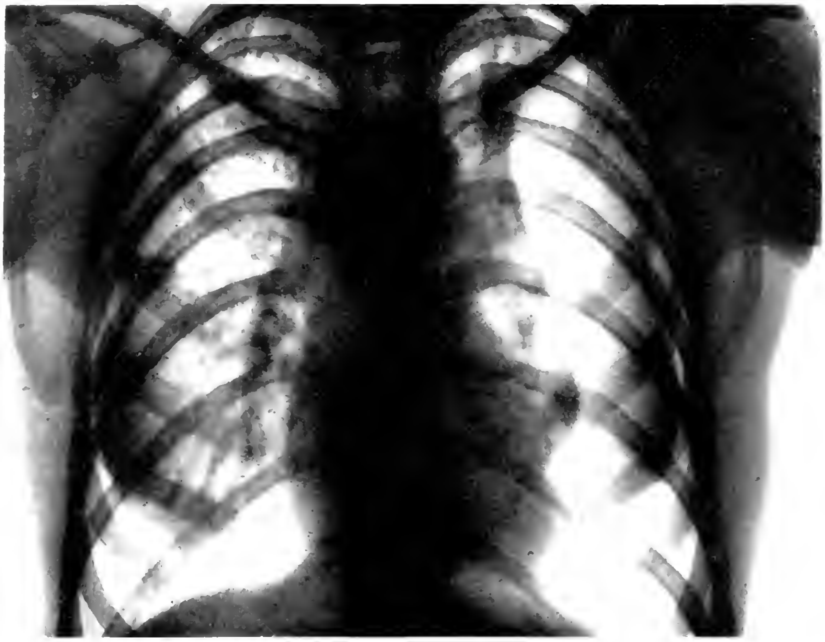


FIG. 1.

FIG. 2.

(Means and Balboni: Factors of Respiration in Pneumothorax.)

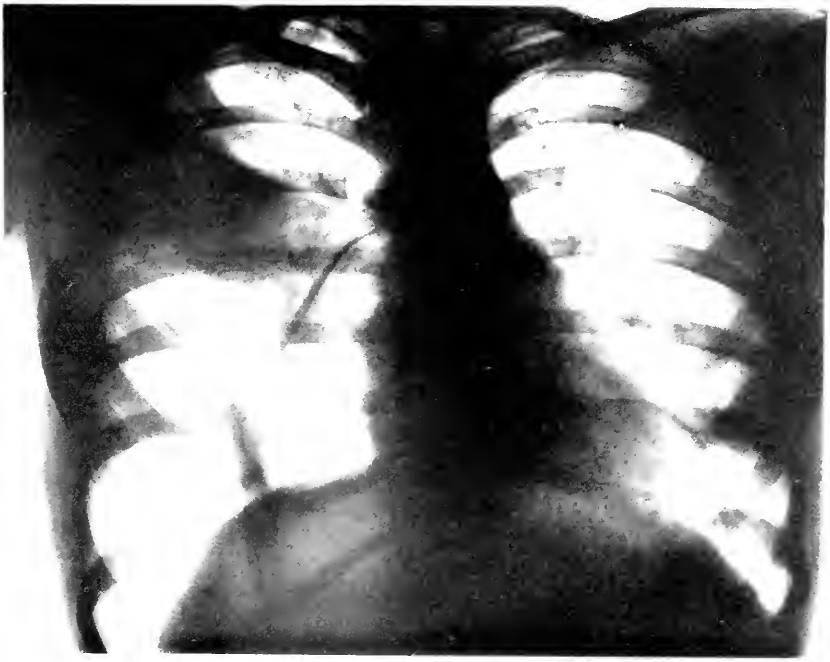


Fig. 3.

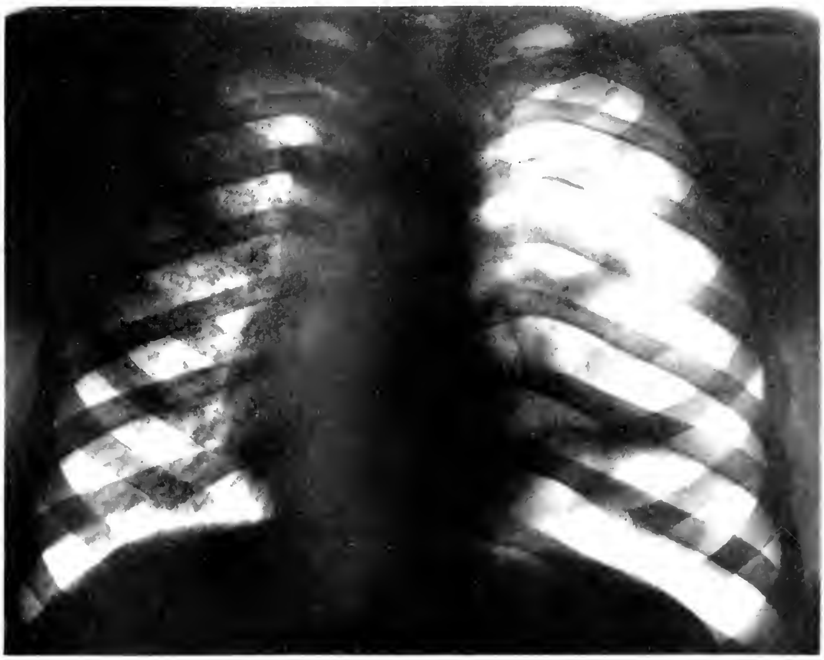
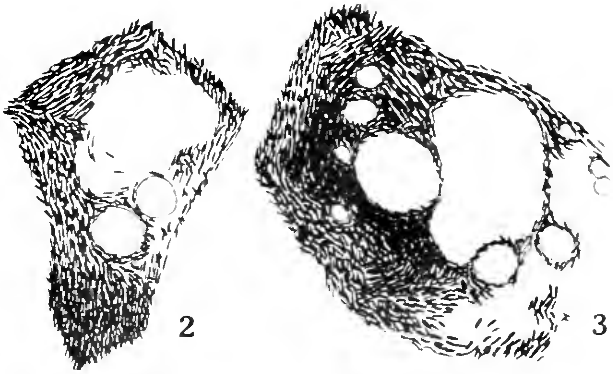


Fig. 4

(Means and Balboni: Factors of Respiration in Pneumothorax.

1

(Uhlenhuth: Changes in Cells of *Rana pipiens*.)



2 3

4 5 6

(Uhlenhuth: Changes in Cells of *Rana pipiens*.)

7 8 9 10

Uhlenhuth: Changes in Cells in *Rana pipiens*

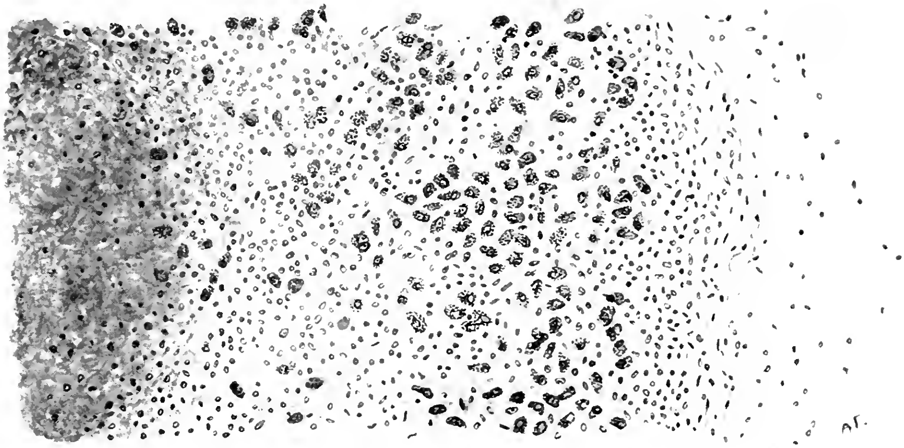


FIG. 1.

FIG. 2.

Cecil: Non Hemolytic Streptococci [illegible]

FIG. 3.

(Cecil: Non-Hemolytic Streptococcus Lesions.)

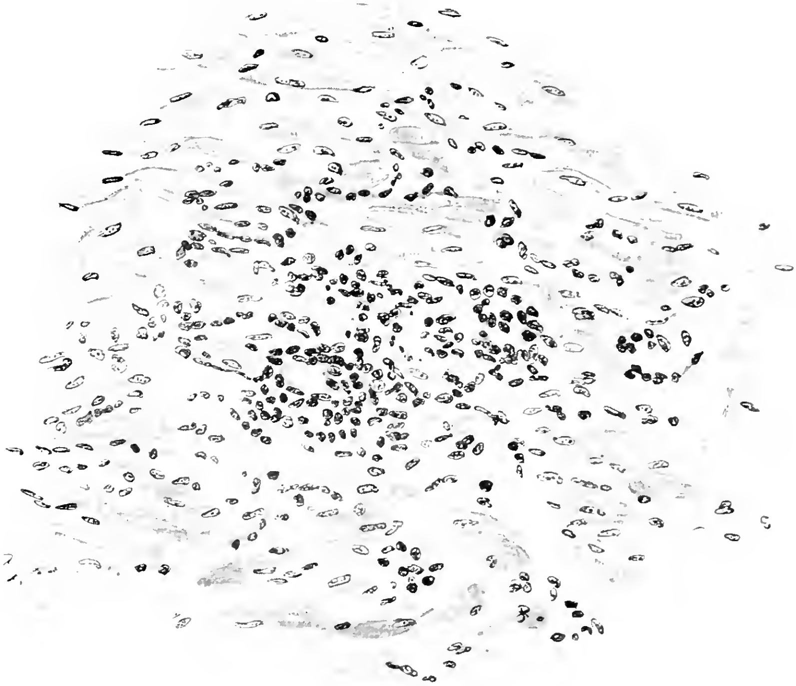


FIG. 4.

FIG. 5

(Cecil: Non-Hemolytic Streptococcus Lesions.)